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Signal Exchange in Plant-Microbe Interactions

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INTRODUCTION

The specificity of many plant-microbe interactions implies some mechanism of recognition, one of which may be the exchange of molecular signals between host and microbe. Such signal molecules can have either a positive or a negative effect on the plant-microbe interaction. Specific cell-cell interactions involve the recognition of a cell or signal molecule or both by another cell. A microbe's recognition of the host's cell surface or a component of it could induce the physiological responses necessary for the interaction. Conversely, recognition of a microbe by a host may induce the expression of genes necessary to defend against or enhance the interaction. In general, recognition is the culmination of a complex series of events.

The exchange of signal molecules appears to be necessary

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for many plant-microbe interactions. The purpose of this article is to identify the signal molecules involved in plant-microbe interactions and the physiological role that these molecules play. Signal exchange in plant-microbe interaction involves the recognition and exchange of specific molecules by the host or microbe or both that trigger biochemical, physiological, or morphological responses that affect the development of the plant-microbe interaction. The signal molecules may be deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, lipid, or polysaccharides. Successful interactions depend on signals that initiate the process and, further, signals that maintain and control the interaction.

This article will consider three examples of plant-microbe interaction: (i) phytoalexins and their elicitors, (ii) *Agrobacterium* spp. and the crown gall disease of dicotyledenous plants, and (iii) the *Rhizobium*-legume symbiosis. These three systems are fairly well characterized and provide diverse examples of plant-microbe interactions.

The signal molecules involved in the establishment of plant defense responses are just beginning to be identified. Plants defend themselves from microbial pathogens through several different mechanisms, including the synthesis of antimicrobial phytoalexins, the hypersensitive response, and cell wall modification. Conversely, recognition of a host by the pathogen can induce the development of parasitic forms of the pathogen.

In a number of host/pathogen systems, there is ample genetic evidence for the existence of a gene-for-gene relationship between host and pathogen (90). For each gene in the host that governs resistance, there is a corresponding gene in the pathogen that governs specific avirulence for that host. Resistance in the host is usually dominant, as is avirulence in the pathogen. That is, resistance and avirulence require gene expression; sensitivity and virulence can arise from the loss of gene function. Race-cultivar-specific compatibility arises when the genetic determinants of the host and parasite do not correspond. The avirulence genes of a pathogen may encode or control the synthesis of molecules that can bind to receptors in the plant and activate defense mechanisms. Immediate induction of host defense responses results in an incompatible reaction. The plant's receptors might be products of resistance genes. Binding to these receptors activates expression of the genes involved in resistance (219). Genetic changes in race-cultivar interactions may be at regulatory loci rather than in structural genes. Activation (or inactivation) of the regulatory loci would regulate expression of the structural genes associated with disease resistance (237).

In general, the exchange of signal molecules between plant host and pathogen is complex and not well characterized, but among these, phytoalexins and their elicitors are the best characterized.

PLANT DISEASE RESISTANCE

Phytoalexin synthesis is not the only disease resistance mechanism used by plants, nor is it always an entirely effective mechanism for resistance. Rapid cell death in the vicinity of the infection site, a response called the hypersensitive reaction, is a common defense response (162). The hypersensitive reaction in host cells is characterized by water loss, a rapid collapse of the cell, bleaching, and the cell flattening (220). The invading microorganisms are effectively contained within the collapsed area, thus protecting the surrounding healthy tissue. The mechanism(s) whereby the hypersensitive reaction is induced by the invading microorganisms is unknown. The hypersensitive reaction has been recently reviewed by Sequira (220).

Modification of the cell wall is another mechanism used by plants to defend against microbial pathogens. The hydroxyproline-rich glycoprotein content of the cell wall in the area of the plant directly affected by the pathogen can change in response to a potential pathogen (88, 104). Increases in hydroxyproline-rich glycoprotein are directly correlated to a greater resistance to the pathogen. Plants that are more susceptible to the pathogen do not respond by increasing the hydroxyproline-rich glycoprotein content of their cell wall. The lignin content of the plant cell wall also changes in response to a potential pathogen (104). Changes in the plant cell wall may inhibit passage of the pathogen.

Plant disease resistance is apparently dependent upon the interaction of more than one resistance mechanism to inhibit invasion of the pathogen. For example, the hypersensitive reaction can occur concurrently with the synthesis of antimicrobial phytoalexins. However, both plant defense responses can operate independently.

Phytoalexins. The occurrence of phytoalexins in plant cells in response to an infection by pathogenic fungi or bacteria is believed to be an important factor in plant disease resistance. Phytoalexins are a chemically heterogeneous group of low-molecular-weight compounds with antimicrobial properties (161). Phytoalexins are not present in healthy plant tissues, but they appear at the site of an infection (20, 166). Phytoalexins synthesis can be induced by molecules of abiotic or biotic origin called elicitors. These include fungal β-glucan (11, 15), glycoprotein (76, 132, 146), and lipid (13, 36) cell wall components. Endopolygalacturonase, isolated from both fungal (146, 147) and bacterial (58) cell walls, functions as an elicitor by releasing an endogenous elicitor from the plant cell wall. This endogenous elicitor activity is mediated by pectic fragments released from the plant cell wall as a consequence of the action of microbial enzymes (146, 147). The presence of an endogenous elicitor in plant cell walls may be the mechanism by which abiotic molecules express elicitor activity (57). Thus, plants have the capacity to recognize molecular signals, originating from both microbes and their own cell walls, which elicit phytoalexins as part of their defense response.

Host and pathogen protein factors. Lectins may play a role in plant disease resistance. Lectins are noncatalytic proteins which interact specifically with carbohydrate residues. Lectins may function as important effectors in the host defense response. For example, soybean lectin (SBL) has been implicated in the resistance response to infection by *Phytophthora megasperma* f.sp. *sojae* (95). The quantity of SBL is greater in cultivars resistant to *P. megasperma* than in susceptible cultivars. Furthermore, seed lectin is released sooner and at higher concentrations into the surrounding media during germination from resistant cultivars than from susceptible cultivars. Following release, the lectin retains its hemagglutinating activity. SBL binding to the mycelial cell surface is inhibited by its hapten, D-galactose. Purified SBL inhibits mycelial growth at relatively high concentrations (0.3 to 0.78 μg of SBL per 100 μl [2.5×10^{-6} to 6.5×10^{-6} M] of a 10^6-zoospore/ml fungal suspension) (95). Wheat germ agglutinin has been implicated as a mechanism of resistance to *Tricoderma viride*. Wheat germ agglutinin binds exclusively to hyphal tips and septa of *T. viride* (179). Binding is inhibited by the hapten chitotriose. Growth and spore germination of *T. viride* are visibly inhibited by wheat germ agglutinin at a concentration of 30 μg in 50-μl (3.3×10^{-5} M) wells on a potato dextrose agar plate (179).

The avirulent *Pseudomonas solanacearum* strain B1 attaches to suspension-cultured tobacco cells and tobacco leaf cell walls and is strongly agglutinated by a hydroxyproline-rich glycoprotein extracted from potato tubers (84, 145, 221). In contrast, the virulent strain K60 is weakly agglutinated by the potato hydroxyproline-rich glycoprotein and binds poorly to tobacco cell suspension cultures and tobacco leaf cell walls (84, 221). The avirulent mutants of *Pseudomonas solanacearum* lack the O-antigen oligosaccharide of the cell wall lipopolysaccharide (LPS) (106). Attachment of the bacteria to the plant cell by the core region of the LPS may be necessary for initiation of the hypersensitive response. The presence of the O antigen in virulent strains may prevent it from binding to the plant cell wall receptor. The physiological role the hydroxyproline-rich glycoprotein plays in the defense response has not been elucidated.

Plant cell wall modification. Modification of the glycoprotein content of the plant cell wall is another plant defense response to a pathogen. The concentration of hydroxyproline-rich glycoproteins in the regions of the plant cell wall affected by disease increases when infections occurs. This increase in glycoprotein level correlates with an increased resistance; conversely, a reduced level correlates with an increased susceptibility to infection (104). Hydroxyproline-rich glycoproteins have agglutinating properties and may inhibit passage of the pathogen through the cell wall. *Collectotrichum lagenarium* inoculation onto musk melon causes a 10-fold increase in the amount of plant cell wall hydroxyproline-rich glycoprotein (88). This increase is a direct result of ethylene biosynthesis caused by plant wounding (87, 241). Ethylene is released from plants upon injury or stress. Ethylene also is thought to play a role in plant senescence (225). Treatment of melon hypocotyls or petioles with an elicitor from *C. lagenarium* causes an immediate inhibition of protein synthesis and, after 18 h, induces the synthesis of plant cell wall hydroxyproline-rich glycoprotein (212). Ethylene content increases early in elicitor-treated plant material. The presence of aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis, inhibits elicitor-induced hydroxyproline-rich glycoprotein (212). Recently, a low-molecular-weight fungal glycopeptide elicitor of ethylene synthesis in melon has been isolated from the *C. lagenarium* mycelium cell wall and culture filtrates. Three different glycopeptides elicitors were identified which differ only in their relative content of sugar, amino acid, and phosphate residues (240). Treatment of the elicitor with weakly alkaline reagents (N_2H_5OH [hydrazine] or NaOH-$NaBH_4$) alters the sugar moiety and reduces its elicitor activity. Thus the activity appears to reside in the carbohydrate moieties. Elicitors isolated from *Phytophthora* spp. (*P. megasperma*, *P. capsici*, *P. parasitica*) induce ethylene biosynthesis in their hosts as well as in the nonhost, melon. The elicitors produced are neither species specific nor ethylene specific, because the *P. megasperma* elicitor induces the synthesis of both ethylene and phytoalexins in soybeans (7, 240). The hydroxyproline-rich glycoprotein content in the cell walls of cucumber cultivars resistant to *Cladosporium cucumerinum* increases 12 to 18 h after inoculation with this pathogen (104). Cultivars susceptible to *Cladosporium cucumerinum* do not increase their cell wall hydroxyproline-rich glycoprotein content until 48 h after inoculation. Penetration of the host by the pathogen occurs within 18 h in all cultivars (104). Recently, Showalter et al. (228) have shown that messenger RNA (mRNA) homologous to the hydroxyproline-rich glycoprotein gene from tomato increases in bean hypocotyls treated with the fungus *C. lindemuthianum*, the causal agent of anthracnose. In a resistant host cross-hybridizing mRNA increases early, whereas in a susceptible host its increase is delayed until the onset of lesion formation. A similar response was obtained upon fungal elicitor treatment of suspension-cultured bean cells. These data suggest that changes in the cell wall structure is another mechanism by which plants are protected against microbial pathogens. These changes in the cell wall are triggered by pathogen-produced factors.

Elicitors of Phytoalexin Synthesis

Numerous molecules have been implicated in mediating disease resistance. Several elicitors of phytoalexin synthesis also induce the expression of other host plant defense responses (e.g., proteinase inhibitor synthesis and accumulation of hydroxyproline-rich glycoproteins). Elicitors of phytoalexin accumulation may be of biotic or abiotic origin. Fungal cell wall β-glucans, glycopeptides, and fatty acids, plant cell wall galacturonide fragments, and microbial pectin-degrading enzymes are examples of biotic elicitors. Heavy metals and ultraviolet (UV) light can function as abiotic elicitors. Phytoalexins and their elicitors have been recently reviewed by Darvill and Albersheim (57).

Arachidonic and eicosapentaenoic acid elicitors. Cell wall extracts of *P. infestans* induce the hypersensitive response and phytoalexin synthesis in potato tubers. Any race of *P. infestans* can induce phytoalexin synthesis in any potato cultivar. The fatty acids, arachidonic and eicosapentaenoic acids, isolated from the *P. infestans* mycelial cell wall have been identifed as the elicitors of phytoalexin synthesis in potato (37). Arachidonic and eicosapentaenoic acids are 20-carbon polyunsaturated fatty acids (36). The concentration of these fatty acids necessary to elicit phytoalexin accumulation is quite high (≥0.3 mM). Therefore, these molecules alone are probably not physiologically important elicitors (37, 57). They account for only 21% of the activity of *P. infestans* mycelial cell wall extract (37). A soluble β-3-, β-6-, and β-3,6-linked glucan oligosaccharide, isolated from the *P. infestans* mycelial cell wall, enhances the activity of arachidonic and eicosapentaenoic acids in the elicitation of the potato phytoalexin rishitin (107, 164, 199) to levels comparable to those caused by the crude mycelium extract (37). Added alone, glucans from *P. infestans* show little or no activity in eliciting phytoalexin synthesis (164, 199). Glucan enhancement of the eliciting activity of arachidonic and eicosapentaenoic acids is not race specific (107). Cell wall constituents which alone exhibit no elicitor activity may show it in the presence of the β-glucan, as has been demonstrated with 20-carbon unsaturated fatty acids (199). The ability of the glucan fractions to enhance the elicitor activity of arachidonic and eicosapentaenoic acids should make it easier to elucidate the important structural components of these fatty acids.

The mechanism by which the β-glucan enhances phytoalexin synthesis by arachidonic or eicosapentaenoic acid is not known. Glucan treatment of potato tubers does not alter the levels of phytoalexin accumulated, inhibit steroid glycoalkaloid accumulation, or induce the hypersensitive response (199). Steroid glycoalkaloids are formed from the condensation of two farnesyl pyrophosphate molecules (199). At this same point, farnesyl pyrophosphate can be used for the synthesis of sesquiterpenoid-derived phytoalexins. Regardless of when the potato disk is treated with the β-glucan fraction (from 18 h before to 24 h after the fatty acid elicitor), there is a concomitant enhancement of

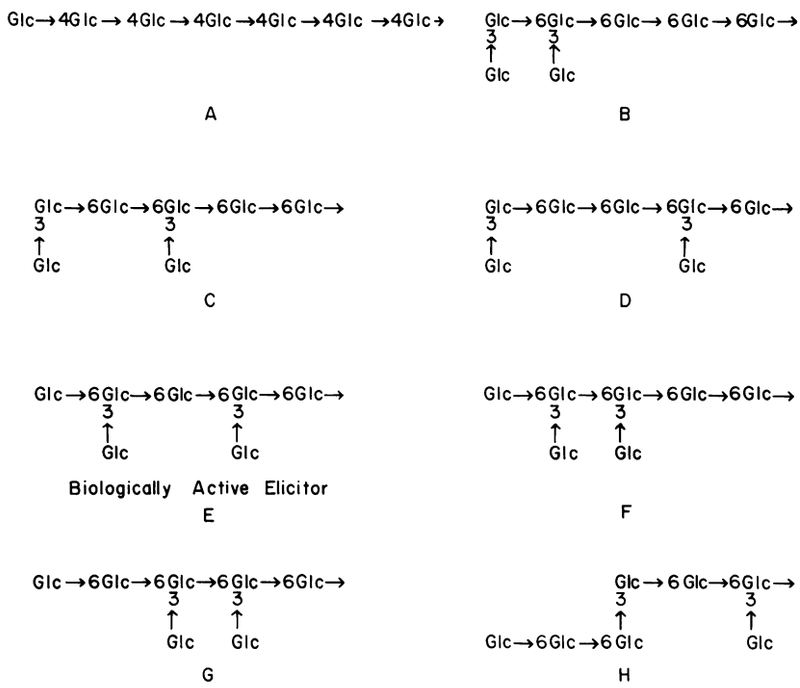

FIG. 1. Structure of the smallest biologically active β-glucan elicitor of phytoalexin synthesis and its biologically inactive isomers. The biologically active hepta-β-glucoside alditol elicitor is shown in (E). The biologically inactive hepta-β-glucoside alditol isomers are shown in (A–D) and (F–H). (Adapted from the figures in references 57 and 223.)

phytoalexin synthesis. Preisig and Kuc (199) suggested that the β-glucan may function by regulating the induction of the physiological response. Knowledge of the effect of β-3,6-glucan on mRNA synthesis in potato tubers with and without the fatty acid elicitors would determine whether the enhancement of phytoalexin synthesis is due to a transcriptional or a post-transcriptional response. Furthermore, experiments are needed to determine the effect of β-glucan on the levels of the enzymes necessary for sesquiterpenoid phytoalexin biosynthesis (e.g., enzymes involved in the melavonic acid biosynthesis pathway and sesquiterpene biosynthesis).

β-Glucan elicitors. Polysaccharide fractions isolated from culture filtrates or from cell walls of *P. megasperma* f.sp. *glycinia* have been implicated as elicitors of the phytoalexin glyceollin on soybean cotyledons (15–17). The elicitor from the mycelial wall was isolated by partial acid hydrolysis (15). Analysis of the cell wall polysaccharides indicates that the active elicitor consisted primarily of β-3-, β-6-, and β-3,6-glucans (7, 16). The β-glucans isolated from *P. infestans* and *P. megasperma* are structurally similar. The biological difference, however, is the ability to induce phytoalexin synthesis in these microbes' respective hosts. The β-glucans can induce phytoalexin synthesis in soybean cotyledons at a concentration of 10^{-8} to 10^{-9} M (225), but show little or no phytoalexin synthesis in potato tubers even at higher concentrations (199).

Mycelial cell wall polysaccharide fractions were isolated from races 1, 2, and 3 of *P. megasperma*. Nearly identical polysaccharide fractions were isolated from each race of *P. megasperma* with almost indistinguishable elicitor activity (16). Phenylalanine ammonia-lyase (PAL) activity increases significantly in both Harasoy and Harasoy 63 cell suspension cultures (16) when challenged with the β-glucan fraction of race 1 (16, 17). PAL is an enzyme of the general phenylpropanoid metabolism pathway which is involved in the synthesis of phytoalexins (see Fig. 2). Soybean cultivars Harasoy and Harasoy 63 are incompatible and compatible with race 1, respectively. The β-glucan from race 1 (17) stimulated PAL activity in parsley and sycamore cell cultures (16). β-Glucan elicitors from the mycelial cell wall of *P. megasperma* also induce phytoalexin accumulation in kidney bean (52). The β-glucan elicitor from *P. megasperma* induces the same five antifungal compounds in kidney bean as does the kidney bean fungal pathogen *C. lindemuthianum* (52). Anderson (11) isolated β-glucans from three different species of *Colleotrichum* that elicited the hypersensitive response and phytoalexin accumulation in kidney bean. Two species were nonpathogenic to kidney bean, while the third was pathogenic. The data indicate that there is very little specificity associated with the host response to fungal β-glucans. Therefore, the β-glucan elicitors are most likely not the primary determinants of race- or species-specific resistance. This is contrary to the earlier work of Keen, who suggested that the *P. megasperma* glucan elicitors were race specific (131). Plants may respond to a variety of fungal pathogens by recognizing the same or similar mycelial cell wall components (i.e., β-glucans), but this alone would not mediate race specificity.

As mentioned above, the structure of the β-glucan elicitor isolated from *P. megasperma* cell walls was identified as β-3-, β-6-, and β-3,6-linked glucans (7, 17). Previously, branching of the β-glucan oligosaccharides was shown to be necessary for elicitor activity; unbranched β-3,6-glucans have been demonstrated to exhibit little or no elicitor activity (7, 57). The molecular structure of the smallest β-glucan oligosaccharide from *P. megasperma* isolated from mycelial cell walls, exhibiting elicitor activity, has been determined (224) (Fig. 1). Partial acid hydrolysis of *P. megasperma* mycelial cell walls releases the active elicitor β-glucan

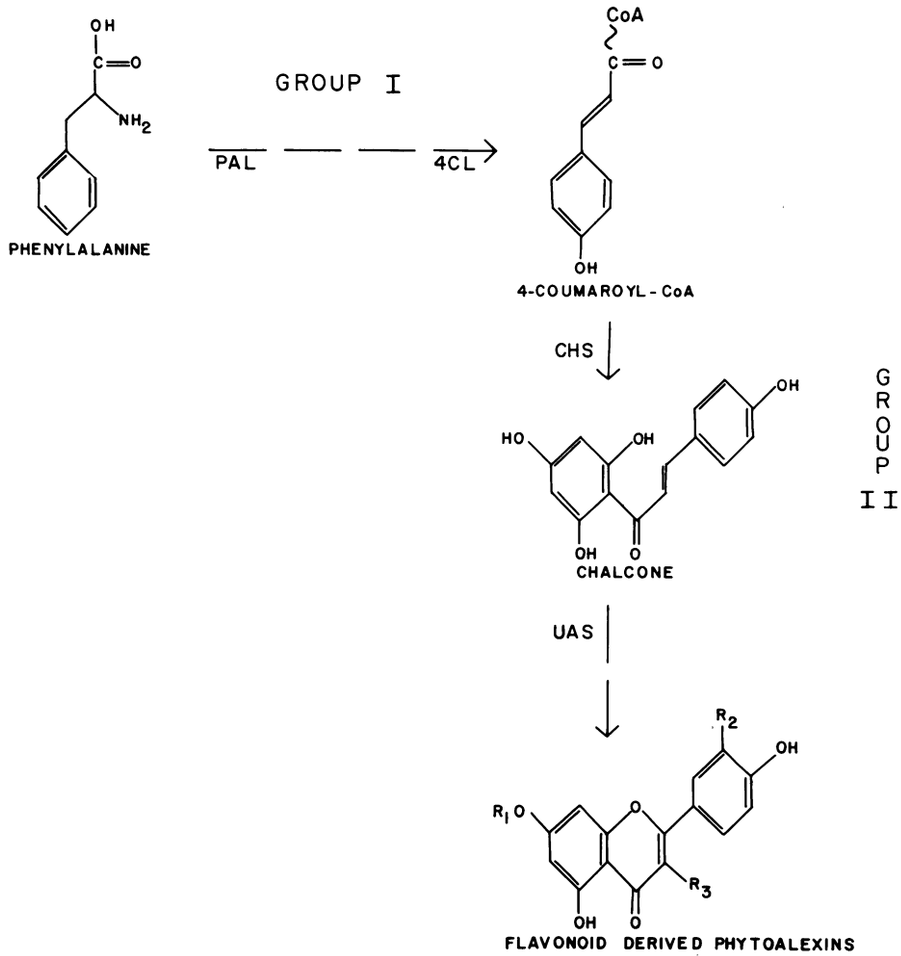

FIG. 2. Schematic diagram of the proposed pathways for the biosynthesis of isoflavonoid-derived phytoalexins. The general phenyl propanoid metabolism pathway (group I) includes the enzymes PAL and 4-coumorate:coenzyme A (CoA) ligase (4CL). The flavonoid glycoside pathway (group II) consists of 13 enzymes, including CHS and uridine 5'-diphosphate (UDP)-apiose synthase (UAS).

oligosaccharide. During the purification procedure, the active β-glucan oligosaccharide elicitor was monitored by the soybean cotyledon assay. The β-glucan fraction was purified by gel filtration and normal and reversed-phase high-pressure liquid chromatography (225). Purification of the β-glucan oligosaccharides results in the isolation of eight hepta-β-glucoside-alditol isomers (225); only one of the eight isomers exhibits elicitor activity (Fig. 1). The molecular structure of the biologically active hepta-β-glucoside alditol elicitor has been determined and confirmed by chemical synthesis (223). Only 0.6 ng (10^{-8} to 10^{-10} M), under the assay conditions used, of the biologically active hepta-β-glucoside elicitor per cotyledon is necessary to elicit a half-maximal response of phytoalexin synthesis (225). The seven hepta-β-glucoside-alditol isomers exhibited no elicitor activity at concentrations 25 times that of the biologically active form (225). The chemically synthesized hepta-β-glucoside-alditol elicitor exhibits the same elicitor activity as the purified elicitor (223).

Hepta-β-glucoside-alditol isomers of the biologically active hepta-β-glucoside elicitor have been structurally characterized and are shown in Fig. 1. The biologically active hepta-β-glucoside alditol elicitor is characterized by a β-linked glucosyl backbone with two β-glucosyl side chains. The branched residues must be separated by a single backbone residue (Fig. 1) (223, 224). The slightest variation in the location of the branched residues renders the β-glucan biologically inactive. Attachment of a β-linked glucosyl residue to the terminal nonreducing residue diminishes its elicitor activity. The number of β-linked glucosyl residues that can be attached to the reducing terminus of the backbone without affecting elicitor activity has not been determined (224). The smallest fungal mycelial cell wall β-glucan oligosaccharide exhibiting elicitor activity, other than from *P. megasperma* isolates, is yet to be determined. As mentioned previously, β-glucans do not exhibit race specificity or species specificity in eliciting phytoalexin synthesis. Therefore, it is likely that the smallest active elicitor structure of these β-glucans would probably not be different from that of the hepta-β-glucoside-alditol isolated from *P. megasperma*. Experiments are needed to test this prediction. Even though no race/species specificity is exhibited by β-glucans in eliciting phytoalexin synthesis, there are specific constraints on the molecular structure necessary for biological activity. This suggests that the elicitor binds to a highly specific receptor.

Plant cell walls contain β-glucanases and β-glucosidases which have been implicated as part of the plant's defense

response. Soybean cotyledons contain β-1,3-endoglucanases (50, 51, 277) and a β-glucosidase (50) which can degrade β-1,3-glucans (50) from the cell wall of *P. megasperma*. The β-1,3-endoglucanases can degrade β-1,3-glucans (e.g., mycolaminaran, laminarin, carboxymethyl-pachyman) in a random manner, but cannot degrade β-1,6-glucans (e.g., pustalan) or β-1,4-glucans (133). The role of plant β-1,3-endoglucanases and β-glucosidases may involve the release of β-1,3-glucan elicitors from the fungal cell wall. These plant enzymes might function by degrading larger β-glucans into elicitor-active hepta-β-glucoside residues which are capable of eliciting phytoalexin synthesis.

Potato protoplasts are agglutinated and killed by *P. infestans* cell wall glucans (196). More recently, the elicitor-active β-1,3-glucan mycolaminarian was found to bind specifically to isolated soybean membranes (278). These data suggest that the receptor for the β-glucan elicitors is located on plant plasma membranes. The enzymatic activity of the β-1,3-glucanases and β-glucosidase may function by degrading the fungal β-1,3-glucans into elicitor-active fragments which permeate through the plant cell wall to the receptor on the plasma membrane. These enzymes may also function to degrade β-glucan elicitors and thus to localize their effect (57). This could minimize the passage of β-glucan elicitors within the plant and the induction of phytoalexin synthesis in areas not involved in the microbial invasion (57). Infection of immature pea pods with *Fusarium solani* f.sp. *phaseoli* (nonpathogenic) and *F. solani* f.sp. *pisi* (pathogenic) induces β-1,3-glucanase and chitinase activities to levels four and nine times greater, respectively, than the uninfected controls (175). The elicitation of β-1,3-glucanase by the nonpathogenic fungi *F. solani* f.sp. *phaseoli* suggests that the induction of pectin-degrading enzymes is a general defense response and not a race-specific defense response.

Plant cell wall fragments that regulate gene expression. *Phytoalexins and proteinase inhibitors*. Plant pectic polysaccharides have been implicated in the induction of two different host defense responses. Proteinase inhibitors I and II and phytoalexin synthesis are induced by α-4-linked galactosyluronic acid residues (38, 188, 261, 262). The elicitors are enzymatically released from plant cell walls by microbial α-1,4-endopolygalacturonases (31, 38, 146, 147). Proteinase inhibitors I and II inhibit the activity of serine endopeptidases found in both invertebrates and microorganisms. The inhibitors are believed to take part in the plants' defense response by decreasing the nutritional quality of plant tissue (213). The proteinase inhibitors function as part of a rapid systemic plant defense response, whereas phytoalexins are part of a localized response.

The size of the α-4-galactosyluronic acid residue may play an important role in regulating the expression of phytoalexin or protease inhibitor synthesis. The "endogenous" phytoalexin elicitors, isolated from citrus pectin and soybean cell walls, are α-1,4-dodecagalacturonides (degree of polymerization [DP] = 12; 188). Smaller galacturonide fragments (DP < 12) are not capable of inducing phytoalexin accumulation. Proteinase inhibitors, however, can be induced with oligogalacturonide fragments varying in size from DP = 2 to 6 to DP = 20 to 30 in tomato (81, 213). Ryan and colleagues (213) suggested that the smaller oligogalacturonides are the signals for proteinase inhibitor synthesis, whereas fragments larger than DP = 10 are signals for phytoalexin synthesis. The larger oligogalacturonides (DP = 20 to 30), which induce proteinase inhibitor synthesis, may not exhibit optimal activity. These large oligogalacturonides may be fragmented further by endogenous polygalacturonases to smaller and more active oligogalacturonides (31). Recognition systems for the endogenous oligogalacturonides elicitors are presumably present in each host to initiate their respective defense response to microbial pathogens.

The endogenous oligogalacturonide elicitor is not nearly as active in eliciting phytoalexin accumulation in the soybean cotyledon assay as is the hepta-β-glucoside elicitor. The hepta-β-glucoside elicitor exhibits a specific activity approximately 1,000 times greater than that of the oligogalacturonide elicitor (188). However, due to the high polygalacturonide content of the soybean cell wall (approximately 6 mg of polygalacturonide per g, fresh weight; 188), the oligogalacturonide elicitor activity is most likely physiologically significant. Application of the endogenous oligogalacturonide elicitors and the fungal hepta-β-glucoside elicitor together produces a dramatic synergistic effect. This synergism is analogous to the role β-glucans play in enhancing the elicitation of phytoalexin synthesis by arachadonic and eicosapentaenoic acids in potato tubers. Applied individually, the partially purified β-glucan and oligogalacturonide do not induce significant levels of phytoalexin (57). Together, approximately 50-fold less β-glucan is required to elicit phytoalexin synthesis (A. G. Darvill, P. Albersheim, M. McNeil, J. M. Lau, W. S. York, T. T. Stevensom, J. Thomas, S. Doares, D. J. Gollin, P. Chelf, and K. Davis, *in* V. Chescenzi, I. C. M. Dea, and S. S. Stivala, ed., *New Developments in Industrial Polysaccharides*, in press). The level of elicitor activity with purified hepta-β-glucoside and the dodeca-α-1,4-oligogalacturonide elicitor would obviously be significantly greater. How the β-glucan and oligogalacturonide elicitors function synergistically is unknown.

Endopolygalacturonases digest the plant cell wall and release oligogalacturonides, which can induce phytoalexin synthesis. A systematic study is needed to establish a correlation between pathogenicity and the presence (absence) of endopolygalacturonases. An incompatible race could induce the activity of both elicitors with the initiation of a more dramatic defense response. A greater level of antimicrobial phytoalexins would be synthesized due to the synergistic effect of the two elicitors. The compatible race may not induce the synergistic response, and therefore a lower level of phytoalexin synthesis would occur. The compatible race could tolerate, detoxify, or degrade the lower levels of phytoalexin.

The role of the β-glucan elicitor in soybean and potato appears to differ. In the soybean cotyledon assay, the β-glucan elicitor is 1,000 times more effective in eliciting phytoalexin synthesis than the oligogalacturonide elicitor. In soybean, the β-glucan and the α-1,4-oligogalacturonide both act as elicitors of phytoalexin synthesis and act synergistically. In contrast, β-glucan elicits little or no phytoalexin synthesis in potato. In potato, β-glucan is not an elicitor but amplifies the effect of the elicitors arachidonic and eicosapentaenoic acids. Apparently soybean and potato both have receptors for β-glucan. The binding of β-glucan to soybean or potato, however, induces a different physiological response.

Oligosaccharins. Naturally occurring plant cell wall carbohydrates exhibiting biological regulatory functions have been termed oligosaccharins (6, 57). Thus the β-glucans and oligogalacturonides discussed above would be classified as endogenous oligosaccharins. Microbial interactions with the plant cell wall may induce the release of oligosaccharins, resulting in the elicitation of host defense responses. Plant cell wall oligosaccharides have also been implicated in the induction of physiological responses other than host defense

responses. Plant cell wall fragments have been shown to (i) inhibit flowering and promote vegetative growth in *Lemna gibba* (98), (ii) inhibit 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments (275), and (iii) control morphogenesis of tobacco explants (242). Partial acid hydrolysis of sycamore suspension-cultured cells and *Lemna* cell walls releases cell wall fragments that would inhibit flowering in *L. gibba* by 60 to 100% (98). The same plant cell wall fragments enhance the number of *L. gibba* fronds by 130 to 300% (98). The rate of vegetative growth is measured by the number of fronds. Neither fragmented nor unfragmented dextran T-10, an α-1,6-linked glucan, had an effect on flowering or vegetative growth. The biologically active fragment is suggested to be a pectic polysaccharide (59, 63). Further characterization of the biologically active fragment and the extension of similar studies to other flowering plants are needed.

Xyloglucan cell wall fragments were isolated from suspension-cultured sycamore cells by an endo-β-1,4-glucanase isolated from *T. viride* culture filtrates (275). A purified xyloglucan nonasaccharide fraction inhibits 2,4-dichlorophenoxyacetic acid-stimulated elongation of etiolated pea stem sections (275). The optimal concentration of inhibition (3.3×10^{-7} to 10^{-8} M) is 100-fold less than that of the auxin analog 2,4-dichlorophenoxyacetic acid. The exact physiological role the nonasaccharide xyloglucan has in inhibiting auxin-stimulated growth is unknown.

Tobacco morphogenesis can be controlled by altering concentrations of the growth regulators auxin and cytokinin or by varying the pH of the culture medium. The effect of plant cell wall fragments on tobacco morphogenesis has been examined. Two different media were used in these experiments. These media differ in the concentration of indole butyric acid (auxin analog), but have the same concentration of kinetin (cytokinin; 5×10^{-7} M). Medium I contains 5×10^{-7} M indole butyric acid and medium II contains 3×10^{-6} M indole butyric acid (242). In medium I, at pH 3.8, an oligogalacturonide cell wall fraction inhibits flowering by 67% with a concomitant induction of vegetative buds on the explants (242). Large amounts of undifferentiated callus are formed at pH 5.0 in medium II without the oligogalacturonide fraction. The addition of the oligogalacturonic acid cell wall fragments inhibits callus formation, and a large number of vegetative buds are formed (242). In medium II at pH 6.0 the explants generally form vegetative buds. In the presence of the cell wall fragments, however, flower formation is induced and bud formation is almost completely inhibited (242). The data support the hypothesis that oligosaccharins regulate morphogenesis in plants. In conjunction with the previously discussed data, a picture emerges in which oligosaccharins play a role as diffusable mediators of plant cell physiology.

Microbial enzyme elicitors. Pectic polysaccharide-degrading enzymes secreted by the pathogen *Rhizopus stolonifer* (38, 146, 147) and *Erwinia caratovora* (58) can induce phytoalexin synthesis in castor bean and soybean, respectively. An endopolygalacturonase, isolated from *R. stolonifer* culture filtrates, is a 32,000-molecular-weight mannose-containing glycoprotein (146, 147) which induces accumulation of the castor bean (*Riccinus communis*) phytoalexin, casbene, in seedlings. Elicitor activity is demonstrated because enzymatic activity and elicitor activity are both lost when the glycoprotein is heated (38). Endopolygalacturonase catalyzes the hydrolysis of polygalacturonic acid (PGA) to dimers, trimers, and tetramers of α-1,4-galacturonide (146, 147). The data indicate that the oligogalacturonide oligomers, released via the endopolygalacturonase activity on PGA in the plant cell wall, are the elicitors which induce casbene synthesis (38). Two heat-labile pectin-degrading PGA lyases that elicit phytoalexin accumulation in soybean have been isolated from *E. caratovora* culture filtrates (58). Pectate lyase activity in culture filtrates of *E. carotovora* and *E. chrysanthemi* is induced by citrus pectin and PGA to levels four times greater than the glycerol control (248). The active elicitor of casbene synthesis was demonstrated to be an oligogalacturonide released by enzyme action on the plant cell wall (58). Therefore, initiation of some host plant defenses may be mediated by the recognition of a signal resulting from the degradation of the plant cell wall, rather than by direct recognition of a component of the pathogen.

Induction of Gene Expression in the Pathogen

Ustilago violacea, the smut fungus pathogen of *Silene abba*, produces hyphae in the host, but on laboratory media they grow as yeastlike sporidia and rarely produce hyphae (59, 60). Contact with the host induces the parasitic mycelial stage and the infective hyphae. Day and colleagues (59, 60) reported the isolation of a heat-stable compound in *S. alba* leaf extracts which would induce morphogenesis in *U. violacea* from saprophytic cells to the parasitic mycelial stage on laboratory media. Also, the host plant extracts enhance the growth and development of conjugation pegs during mating (60). All aqueous plant extracts isolated from a compatible host of *Ustilago* spp. induce hyphae and inhibited sporulation in *U. violacea*, *U. scabiosae*, and *U. utriculosae* (59). Most aqueous extracts of nonhost plants (35 of 41) do not induce hypha formation (59). Methanol extracts of host and nonhost leaves, however, are active inducers of the parasitic stages (44). The results indicate that the observed response is neither race nor species specific, but rather applies to the whole taxonomic group. Compatible host plants either have a greater concentration of the active component(s) or have it in a form which is more readily extracted by water. Reverse-phase high-pressure liquid chromatography, UV spectroscopy, and mass spectrometry were performed on root and leaf methanol extracts of *S. alba* and *Pastinaca sativa* to identify the biologically active components (44). Only one biologically active component was isolated, and it was identified as α-tocopherol (vitamin E). Synthetic α-tocopherol at very low concentrations (10^{-7} to 10^{-8} M; 10^{-17} g per cell) was highly active in inducing hyphae (44). The effects of α-tocopherol on *U. violacea* gene expression and protein synthesis as well as the effects of α-tocopherol or analogous factors on other pathogens need to be determined.

Contact of *Phytophthora* sp. zoospores with the plant root surface results in the transition from motile, wall-less cells, through the stage of sessile walled cysts, to germlings which penetrate the host. Zoospores of *P. cinnamomi* and *P. palmivora* are induced by galacturonides to accelerate encystment and germination in vitro (99, 124). Citrus pectin, polygalacturonate, alginate, and galacturonate induce encystment and germination. Water-soluble extracts of maize and lupin root mucilage also induce parasitic development in *P. cinnamomi* (124). The root mucilage may provide the natural signal which causes the induction of parasitic stages in *P. cinnamomi*. Within 5 min of the addition of 500 μg of pectin per ml to a suspension of *P. palmivora* (>90% zoospores), differentiation is almost com-

plete (<5% zoospores) (99). Addition of polygalacturonic acid (150 μg/ml) results in over 80% encystment within 5 min (99). Pectins have a backbone consisting of α-1,4-linked galacturonic acid residues which can be methylated. Polygalacturonates have a similar structure but lack methylation. This suggests that uronic acid residues are the active components for the induction of zoospore differentiation. Root mucilage and surface carbohydrates may be more effective in the induction of encystment than the isolated polygalacturonates. Additional plant products derived from root mucilage may induce parasitic development. Citrus pectin and polygalacturonates may be structurally similar but not identical to the root mucilage inducer. This would explain their reduced biological activity. The inducer from the root mucilage needs to be purified and its biologically active structure needs to be determined to further understand its mode of action.

The presence of a host-specific signal compound(s) which induces nodulation competence in *Bradyrhizobium* spp. has been reported and will be discussed later. The induction of parasitic stages in previously saprophytic *U. violacea* and *Phytophthora* spp. is analogous to the induction of nodulation competence in *Bradyrhizobium* spp. The ability of *U. violacea* and *Phytophthora* spp. to respond to these ubiguitous compounds is consistent with their broad host ranges. Each genus of parasite (symbiont) may respond to an inducer of parasitic differentiation which is ubiquitously found in their hosts. The induction of parasitic stages by species-specific host plant signals in host-pathogen relationships is conceivable. Nonpathogenic or avirulent microorganisms would not be able to recognize and respond to a signal molecule.

Induction of polygalacturonide-degrading enzymes by host cell walls has been observed in several fungal pathogens. However, no correlation between induction of polygalacturonide-degrading enzymes and compatibility/incompatibility in host-pathogen interactions has been demonstrated. Host cell walls induce polygalacturonase activity in *Botrytis fabae*, *Botrytis cinerea*, and *F. oxysporum* f.sp. *lycopersici* when used as a sole carbon source for growth (53). Host cell walls did not induce polygalacturonase activity in *Scelerotina fractigena*. Several nonhost cell walls did not induce polygalacturonase activity in *Botrytis fabae* and *F. oxysporum* f.sp. *pisi* (53). All of the above fungal pathogens are able to cause significant wall degradation in vivo. Substrate induction of pectate lyase activity has been demonstrated in the bacterial pathogens *E. caratovora* and *E. chrysanthemi* (248). The induction of pectin lyase activity in *E. chrysanthemi* in response to added substrate is inhibited by the addition of rifampin. Pectin and pectate lyases of *E. chrysanthemi* are two distinct enzymes which are differentially regulated. Pectate lyase can depolymerize polygalacturonate but not pectin, which can be degraded by pectin lyase. The induction of these pectolytic enzymes by host cell wall components may play an important role in pathogenicity.

The success of some pathogens (compatible races) may rely on their limiting the breakdown of host cell walls and thereby preventing the release of the endogenous galacturonide elicitor. The regulation of the synthesis of pectin- or polygalacturonate-degrading enzymes or both needs to be examined in both compatible and incompatible interactions. Signal molecules, other than the substrate, which induce hydrolytic enzymes need to be identified. Attachment or recognition of the plant cell wall may induce the synthesis of other factors involved in virulence.

Microorganisms capable of metabolizing or detoxifying phytoalexins may have a greater pathogenicity. Compatible pathogens are generally more tolerant to phytoalexins than incompatible pathogens (53, 220, 252). Some compatible pathogens, however, are sensitive to the phytoalexin and cannot degrade it. Metabolism of phytoalexin by a pathogen can be constitutive or induced. The 1-h lag period for the demethylation of pisatin by *Nectria haematecca* is not observed in the degradation of a second dose of pisatin (88). The enzymes necessary for pisatin demethylation, therefore, are likely induced by the phytoalexin. Compounds structurally similar to the phytoalexin pisatin are capable of inducing the degradative enzymes (252). Cycloheximide addition prevents induction of the enzymes necessary for phytoalexin degradation, indicating that de novo protein synthesis is required (135, 253). The lag period for the metabolism of phytoalexins is not exhibited by other pathogens (253). It is not known whether a pathogen's capacity for degradation of phytoalexins could be induced above the constituitive levels. Further study is needed concerning the effect of phytoalexins or other host plant compounds on the pathogen's phytoalexin metabolism.

Lectins have been implicated as determinants of host-pathogen interactions. Lectins agglutinate both bacterial and fungal pathogens. Wheat germ agglutinin binds exclusively to hyphal tips and septa of the wheat pathogen *T. viridae* and inhibits the pathogen's growth and spore germination (179). SBL binds to the mycelial cell wall of *P. megasperma* f.sp. *sojae*, inhibiting mycelial growth (179). Growth is only inhibited by a relatively high concentration of SBL; hence the biological validity of the observation is questionable. Avirulent strains of *Pseudomonas solanacearum* are strongly agglutinated by potato hydroxyproline-rich glycoproteins, whereas virulent strains are not. Avirulent strains of *Pseudomonas solanacearum* stop growing in vivo within 6 h after attachment to tobacco cells (219). The hypersensitive response is not visible within 6 h. Virulent strains grow exponentially until the hypersensitive response is visible 48 h later (219). The above observations suggest that attachment of incompatible or avirulent pathogens to the plant induces a physiological response which inhibits growth and development of the pathogen. A purified lectin from salmon ova exhibits antibacterial activity towards four different bacterial species pathogenic to salmon (260). Growth of the pathogenic bacteria in vitro was prevented by the presence of this lectin (260). How agglutinins inhibit cell growth of plant or animal microbial pathogens needs to be determined. In plants, lectins may induce or repress genes involved in the synthesis of elicitors of the hypersensitive response. The effect of lectin on gene expression and pathogenicity needs to be assessed. Binding of the pathogen to a lectin located in the plant cell wall or plasma membrane may be important in the induction of the hypersensitive response.

Induction of Gene Expression in Plants by Elicitors

Disease resistance involves the induction of defense mechanisms such as phytoalexin synthesis, proteinase inhibitor synthesis, and accumulation of hydroxyproline-rich glycoproteins (57, 88, 213). These defense responses can be induced not only by microbial infection, but also by β-1,3-glucans, oligogalacturonides, and glycoprotein or fatty acid elicitors isolated from microbial cell walls. Disease resistance in pea is associated with increased levels of specific mRNAs after challenge with the root-rotting fungus *Fusarium solani* f.sp. *phaseoli* (211). These mRNAs are most

likely associated with enzymes necessary for phytoalexin synthesis. Evidence for de novo synthesis of both mRNA and the enzymes necessary for phytoalexin synthesis has been obtained in other systems (85, 215, 279). The β-1,3-glucan elicitors isolated from *C. lindemuthianum* cell walls cause a rapid induction of the enzymes necessary for phytoalexin synthesis (144); these glucans stimulate the transcription of the enzyme-encoding genes (52, 213). The induction kinetics of chalcone synthase (CHS) synthesis and CHS mRNA exhibits nearly identical time courses in suspension-cultured *Phaseolus vulgaris* (bean) cells (214). The maximal induction of CHS (55, 214), PAL (55), and chalcone isomerase (55) mRNAs is attained approximately 3 to 4 h after treatment with the elicitor. In vitro translation products of newly synthesized mRNA from elicitor-treated and control cells have been compared (55). Newly synthesized mRNA was separated from preexisting mRNA by organomercurial affinity chromatography after in vivo labeling with 4-thiouridine (55). Following elicitor treatment of the suspension-cultured bean cells, the synthesis of several mRNA species decreases, while a larger number increased. Several mRNA species induced by the elicitor arise from an undetectable or very low basal rate in the control cells. Several of the mRNA species are unaffected by the elicitor (55).

UV light or the β-glucan elicitor isolated from the cell walls of *P. megasperma* induce the transcription of PAL and 4-coumerate:coenzyme A ligase mRNAs in suspension-cultured parsley cells (*Petroselinum hortense*; 93, 100, 140, 208). PAL and 4-coumarate:coenzyme ligase are two of the enzymes involved in general phenylpropanoid metabolism (group I). CHS and uridine 5′-diphosphate-apiose synthase are two of several enzymes involved in the flavonoid glycoside biosynthetic pathway (group II). Figure 2 shows a diagrammatic representation of the pathway for the synthesis of flavonoid-derived phytoalexins. The phytoalexin biosynthetic enzymes are induced by both UV and the fungal β-1,3-glucan in most plants. The rate of induction of group I enzymes is more rapid than that of group II. Treatment with UV induces flavonoid pigment production, partially explaining the induction of CHS and chalcone isomerase activity. However, how abiotic elicitors in general (e.g., UV) mediate the induction of phytoalexin synthesis is unknown. UV light, in addition to inducing pigment production, may also cause wounding or tissue death. Darvill and Albersheim (57) suggested that abiotic elicitors cause cell injury or death which releases cell wall pectic fragments. These plant cell wall fragments could be the same as the endogenous oligogalacturonide elicitors mentioned earlier.

In the soybean cotyledon bioassay, the β-1,3-glucan elicitor from *P. megasperma* is approximately 1,000 times more active than the oligogalacturonide elicitor isolated from plant cell walls. A synergistic response is observed, however, when one combines the β-1,3-glucan elicitor and the pectic oligogalacturonide fragment. Under these conditions the β-glucan elicitor is 50 times more active. The β-1,3-glucan could be inducing group I phytoalexin biosynthetic enzymes and the oligogalacturonide elicitor could be inducing both groups I and II. Induction of both groups could explain the greater activity of the elicitors in the induction of phytoalexin synthesis. The same could be true for the enhanced response of sesquiterpenoid phytoalexin accumulation in potato with β-glucan and arachidonic and eicosapentaenoic acids. Sesquiterpenoid phytoalexins are not synthesized from isoflavonoids but from melavonic acid. A dual induction system could also occur here since β-glucans elicit very little or no phytoalexin synthesis, but rather increase the level of phytoalexins elicited by arachidonic acid by approximately 2.5- to 3-fold (199). Experiments with purified elicitors, fungal β-1,3-glucans, oligogalacturonide pectic fragments, arachidonic and eicosapentaenoic acids, lectins, or glycopeptides would be helpful. These experiments could be used to compare the effect of the elicitors individually or in combination on the induction of newly synthesized mRNAs involved in disease resistance. A useful study would be the effect of different sizes of oligogalacturonides (DP = 2 to 6 or ≥10) on the induction of newly synthesized mRNAs. Smaller oligogalacturonides are involved in the induction of proteinase inhibitors, while larger fragments induce phytoalexin synthesis.

A distinct temporal difference in the induction of CHS mRNA activity is observed in hypocotyls of *Phaseolus vulgaris* challenged with an incompatible (β) or compatible (α) race of *C. lindemuthianum* (20). The incompatible (β) race induces a more rapid and localized induction of CHS mRNAs. The induction of mRNA activity by the incompatible race begins approximately 50 h after inoculation (20). The compatible race induces CHS mRNA activity approximately 120 h after inoculation. Increases in CHS mRNA activity are observed in tissue distant from the site of inoculation with the compatible race (20). The data demonstrate that the induction of CHS mRNA and phytoalexin gene expression is temporally controlled in incompatible/compatible race-cultivar interactions. β-Glucans isolated from mycelial cells of the compatible and incompatible races exhibit almost identical elicitor activities on kidney bean cotyledons (11). The molecular events which distinguish an incompatible from a compatible interaction are unknown. The incompatible strain may have pectin-degrading enzymes (e.g., polygalacturonases) which can degrade the plant cell wall and release an endogenous elicitor, thereby inducing a faster host defense response. Anderson (12) demonstrated, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, distinct differences in the profiles of extracellular glycoproteins produced by compatible and incompatible races of *C. lindemuthianum*. The incompatible and compatible races have two extracellular high-molecular-weight bands in common which migrate together when electrophoresed. The incompatible race has four unique bands. The unique extracellular glycoproteins of the incompatible race may play a role in enhancing the defense response. Extracellular glycoproteins isolated from an incompatible race may have an effect on the mRNA activity in bean hypocotyls. To determine which signal molecule induces or represses gene expression in the defense response, purified phytoalexin elicitor or other microbial gene-regulating molecules need to be used individually and in combination.

Elicitor-treated cell suspension cultures do not exhibit specificity in the induction of phytoalexin synthesis (105). Parsley and sycamore cell suspension cultures synthesize enzymes necessary for phytoalexin synthesis when challenged with an elicitor obtained from fungi nonpathogenic to these plants. Soybean cell cultures, when challenged with the β-glucan elicitor isolated from a compatible or incompatible race of *P. megasperma*, synthesize phytoalexins (16). Suspension-cultured bean cells, when challenged with a β-glucan elicitor from the pathogen, or with the pathogen itself, induce synthesis of mRNA-encoding enzymes of the general phenylpropenoid and isoflavonoid biosynthetic pathways (55, 112). The lag period for the induction of PAL, CHS, and chalcone isomerase is much shorter in cell suspension cultures than in bean hypocotyl sections (54). The

TABLE 1. Signal molecules involved in plant defense responses and microbial pathogenicity

Identified signal molecule	Possible or unidentified signal molecule	Physiological response	Reference(s)
α-Tocopherol		Induces hyphae in *U. violacea*	44, 59, 60
Galacturonic acids	Root mucilage	Induces encystment and hyphae in *Phytophthora* sp.	99, 124
Arachidonic and eicosapentaenoic acids		Phytoalexin synthesis	33, 36, 37, 199
Hepta-β-glucoside alditol (β-3, 6-glucan)		Phytoalexin synthesis	11, 15, 223–225
Ethylene	Fungal glycopeptide	Increases HRGP content of plant cell walls	87, 88, 240, 241
Oligogalacturonide (DP = 2–6 to DP = 20–30)		Proteinase inhibitors I and II	31, 212, 261, 262
Oligogalacturonide (DP ≥ 10)		Phytoalexin synthesis	146, 147, 188
Microbial pectin-degrading enzymes		Phytoalexin synthesis	58, 146, 147, 248
	Fungal extracellular glycoproteins	Compatibility/incompatibility determination	12

lack of secondary cell wall structures in cell suspension cultures makes these systems much different from studies with whole plants. For example, the delay observed in induction of phytoalexin synthesis in bean hypocotyls is likely due to the time it takes for the fungal spore to germinate, penetrate the cuticle, and come in contact with the first host cell. The advantages and disadvantages of tissue cultures, cell suspension cultures, and protoplasts in studying host-pathogen interactions have been recently reviewed (208).

Concluding Remarks

A list of identified signal molecules and possible unidentified signal molecules involved in the plant defense response or microbial pathogenicity is given in Table 1. The host plant may depend on its ability to recognize one, a few, or all of the signal molecules (elicitors) of a pathogen to elicit a successful defense response. The failure of a potential pathogen to send a signal or of the plant to respond to the signal could result in a compatible reaction. Conversely, the inability of a pathogen to respond to a signal molecule may put it at a disadvantage for successfully establishing a pathogenic state.

Though race/cultivar specificity and species specificity are exhibited in nature, laboratory experiments do not always demonstrate this. Fungal β-glucan elicitors have been used to demonstrate de novo synthesis of mRNAs necessary for phytoalexin synthesis. Specificity is not exhibited in the induction of phytoalexin synthesis when using partially purified β-glucan elicitors. The rate of induction of mRNAs for phytoalexin synthesis, however, varies for compatible and incompatible races. The use of isolated elicitors alone is understandably useful in elucidating which genes are expressed by that elicitor. However, analysis of newly synthesized mRNAs in response to multiple elicitors or signal molecules in combination could help to delineate how they regulate the plant's defense response. Hypothetically, a signal molecule isolated from a microbial cell wall may repress a key enzyme which is normally induced by the β-glucan elicitor. Repression of this enzyme could inhibit or delay phytoalexin synthesis. Such a phenomenon could partially explain the differential induction of phytoalexin synthesis exhibited by compatible and incompatible races.

A pathogen's recognition of the molecules which affect virulence has not been extensively examined. The induction of fungal parasitic stages in previously saprophytic cells on laboratory media is an isolated case (58, 59). Nevertheless, this observation can be useful for discussion. If fungal parasitic stages are host induced, other genes involved in pathogenesis could also be host induced. Theoretically, genes could be induced which enhance virulence or reduce the host plant's defense response.

The concentration of elicitors applied in most bioassays is extremely high, especially in the potato bioassay with the fatty acid elicitors arachidonic and eicosapentaenoic acids. The need for high elicitor levels makes it difficult to assess the biological role of such elicitors. Elicitors, however, are likely to act in concert with other mitigating factors. An example of this is the observation of enhanced arachidonic acid elicitor activity when applied with β-glucan fragments. Another example is the observation that plant oligogalacturonide cell wall fragments and fungal β-glucans together act synergistically in the induction of phytoalexin accumulation in soybean.

AGROBACTERIUM-CROWN GALL DISEASE

Microbial interaction with a host generally results in the induction of host genes which are either advantageous or detrimental to the microorganisms. The neoplastic crown gall disease of dicotyledenous plants induced by *Agrobacterium* sp. is an exception. Following infection of a wound site, part of the *Agrobacterium* Ti plasmid DNA, the transfer DNA (T-DNA), is integrated into the plant's chromosomal DNA (48, 268). The T-DNA encodes the genes necessary for the synthesis of opines and for tumor morphology. The opines are a class of compounds which apparently serve as a source of carbon, nitrogen, and energy for the bacteria. Genes for the synthesis of the phytohormones auxin and cytokinin are encoded on the T-DNA. Different concentrations of the phytohormones are believed to affect the morphology of crown gall tumors. The Ti plasmid also encodes genes necessary for opine catabolism and for virulence (*vir*). Transfer of the Ti plasmid from one *Agrobacterium* strain to another is dependent upon the presence of opines either in the tumor environment or in vitro (118, 197). The *vir* region of the Ti plasmid is most likely involved in the early phases of tumor induction. *Agrobacterium rhizogenes* is the causative agent of hairy root disease, which is characterized by a large mass of roots emanating from the infected wound site. Like those mediating *A. tumefaciens*-crown gall, the genes necessary for hairy root disease are encoded on a plasmid (Ri), a portion of which is transferred to the host (267).

Information is limited concerning the mechanisms of T-DNA transfer from *Agrobacterium* sp. and T-DNA integration into the host chromosome. The transfer of the T-DNA most likely involves the *vir* region and is facilitated by the

exchange of chemical signals. *Agrobacterium* sp. and crown gall disease have been reviewed extensively (111, 127, 169, 184, 236).

Transfer and Integration of the T-DNA into the Plant Genome

The mechanisms whereby the T-DNA is transferred into the host genome are unknown. Attachment of *Agrobacterium* spp. to the plant cell wall is believed to be a prerequisite for virulence (81, 82). *Agrobacterium* spp. have not been commonly observed to enter the plant cell as do rhizobia. Either the entire Ti plasmid or just the T-DNA portion is transported through the bacterial and plant cell membranes. Koukolikova-Nicola et al. (139) reported the presence of a circular intermediate of T-DNA induced by cocultivation with *Nicotiana tabacum* protoplasts. The concentration of these circular intermediates increases with time of cocultivation (139). The obvious implication of this work is that T-DNA is transferred to the plant as a circular molecule. Since this circular T-DNA molecule exists only during cocultivation, some type of cell-cell signal exchange must occur. Apparently, circularization of the T-DNA in the cell occurs after the recognition of a diffusable signal molecule or following attachment to the protoplast. The mechanism of T-DNA excision from the Ti plasmid and circularization of the T-DNA is unknown. Induction of the *vir* genes by plant extracts has been reported (160, 191, 234a, 234b). Transposon mutagenesis of the *virB* region of the Ti plasmid prevents the transfer of the T-DNA into monocotyledenous plants (119). It is possible that the *vir* genes encode circularization and transfer of the T-DNA in response to a potential host.

Expression of T-DNA in the Plant Cell

Expression of the T-DNA is necessary for tumorigenesis and for survival of the agrobacteria. Four genetic loci and eight transcripts have been identified in the T_L-DNA of pTiA6NC (3, 184, 210) and pTiC58 (111, 123). The T_L portion of the T-DNA encodes the following oncogenic functions: (i) the tumor morphology shoot (*tms*) locus (94, 184, 193), (ii) the tumor morphology large (*tml*) locus (94, 184), (iii) the tumor morphology root (*tmr*) locus (94, 184, 193), and (iv) the opine synthetase (*ocs*) locus (184). Oncogenic functions on the Ti plasmid are separate from the genes necessary for T-DNA transfer. Tumor morphology is dependent upon the concentration and balance of the phytohormones auxin and cytokinin (8). The endogenous levels of auxin and cytokinin are regulated by the *tms* and *tmr* loci, respectively (4). Considerable homology is exhibited between different Ti plasmids for the *tms* and *tmr* loci (47, 75).

The *tms* locus encodes two transcripts necessary for the synthesis of the auxin indole-3-acetic acid (124, 136, 216, 238). The transcript 1 gene product of the *tms* locus is a 83,769-dalton protein which exhibits significant homology to the adenine-binding region of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (136). This suggests that the transcript 1 gene product binds adenine as either a substrate or a cofactor (136). The transcript 2 gene product functions by converting indole-3-acetamide to the auxin indoleacetic acid (124, 216, 238). Transcript 1 may encode a protein necessary for the synthesis of indole-3-acetamide (124). Mutations in either transcript 1 or 2 result in the *tms* (shooty tumors) tumor morphology. The involvement of transcripts 1 and 2 of the *tms* locus in auxin synthesis has been further substantiated by complementation studies. Transcript 1 mutants are able to complement transcript 2 mutants and vice versa in vivo (124).

The *tmr* locus (transcript 4) has been implicated in cytokinin biosynthesis (3, 4). Recently, enzymatic evidence has demonstrated that the *tmr* gene product is a dimethylallylpyrophosphate:adenosine 5′-monophosphate transferase (3). Dimethylallylpyrophosphate:adenosine 5′-monophosphate transferase functions by attaching the dimethylallyl side chain to adenosine 5′-monophosphate to form isopentenyladenosine 5′-monophosphate, which is a biologically active cytokinin (3). The nucleotide sequence of the *tmr* gene indicates that a 240-amino-acid protein could be synthesized from this gene (149).

The T-DNA integrated into the plant genome is transcribed by the plant RNA polymerase II and is translated within the plant cell (111, 269). The eucaryotic control elements, "TATA" and "CAAT" boxes, have been found 5′ to the transcription initiation sites of the T-DNA (111, 184). This suggests that direct control of T-DNA gene expression is regulated by the plant cell. Mutations within the *vir* region of the Ti plasmid, however, can have an effect on tumor morphology (i.e., small or delayed tumors) (112, 159). Exogenous indole acetic acid will complement mutations within the T-DNA but not in regions outside of the T-DNA (i.e., *vir* region) (128). The virulence region may encode diffusable factors which indirectly affect tumor morphology. To determine if this is a possibility, the effect of *Agrobacterium* or purified *vir* region gene products or both on host gene expression or T-DNA gene expression needs to be assessed. The cytosine residues of plant DNA are highly methylated (9). T-DNA integrated into the plant genome is also subject to methylation (9, 108). A decrease in T-DNA methylation causes an increase in nopaline synthase activity (108) and an increase in expression of transcripts 1 and 2. Agrobacteria may play a role in regulating the degree of T-DNA methylation as one potential method of gene regulation.

Plant-Induced Expression of the *vir* Genes

Transposon mutagenesis of the Ti plasmid demonstrates the existence of a region physically distinct from the T-DNA which is necessary for virulence (111, 112, 127, 137). The virulence region (Vir region) has not been detected in plant tumor cells (48, 239). Genetic studies demonstrate that complementation occurs in the *trans* configuration and, therefore, the *vir* genes produce diffusable products (112, 125, 159). Seven loci (*virA–F* and *virO*) have been identified in the Vir region (112, 159). Transposon mutagenesis of the Vir region generally results in the loss of virulence (112, 125, 137, 159). The loss of virulence may affect host range (112, 125, 137) or cause an attenuated (or delayed) virulence pattern on some hosts (112, 159). The *vir* genes have been implicated in the transfer of the T-DNA (119). Treatment of the monocotyledenous plants *Chlorophytum capense* and *Narcissus* spp. with a *virB*-mutated Ti plasmid did not result in infection (119). However, the wild type produced small lesions and synthesized opines (119). It is not clear whether the *vir* genes enter the plant cell or just mediate the transfer of the T-DNA.

Recently, the promoter activity of *virC* was shown to be induced by a plant product (191). The *virC* promoter and part of its structural gene were cloned into a vector which contained the complete *lacZ* gene, coding for β-galactosidase, but lacked the promoter/operator region. No β-galactosidase activity is detected in *Escherichia coli* or *A.*

tumefaciens (lacking the Ti plasmid) cells that contain the *virC* promoter:*lacZ* fusion. Root exudates and plant exudates from the dicotyledenous plants *Daucus carota*, *Nicotiana plumbaginifolia*, *Pisum sativum*, and *Vicia hirasta* induce β-galactosidase activity in *Escherichia coli* and *A. tumefaciens* (191). The extracts of monocotyledenous plants exhibit ambiguous results. *Allium cepa* plant extracts induce β-galactosidase activity, whereas *Zea mays* extracts have little stimulatory activity. The *Pisum sativum* inducer of *virC* promoter activity was found to be sensitive to heat, pronase, and trypsin, and insensitive to ribonuclease I, deoxyribonuclease, and phospholipases A_2 and D (191). This suggests that the inducer is either a protein or a glycoprotein. Equilibrium dialysis indicates that the inducer has a relative molecular weight of >7,000 (191).

The induction of the *vir* genes by plant extracts has also been reported by other investigators (160, 234a, 234b). Nester and colleagues have detected a low-molecular-weight factor produced by the plant that induces *vir* gene expression (234b). Lundquist et al. (160) fused a *vir* gene promoter to the chloramphenicol acetyltransferase gene. A low-molecular-weight factor present in host plant extracts induced chloramphenicol acetyltransferase activity. Indoleacetic acid was also active in inducing chloramphenicol acetyltransferase activity. Recently, further work by Kado's group has shown that *vir* gene promoters fused to the luciferous gene are induced by a polyhedric phenol compound present in host plant extracts (C. I. Kado, personal communication). A number of low-molecular-weight phenolic compounds have also been found by Nester's group to induce *vir* promoter activity (personal communication). Most recently, Stachel et al. (234a) have reported the chemical identification of phenolic compounds present in plant exudates the induce *vir* promoter activity. These compounds are acetosyringone (3,5-dimetoxy-4-hydroxyacetophenone) and hydroxyacetosyringone. The chemical identification of the inducer of *vir* gene expression should lead to further studies on the mechanism of this induction. The work demonstrating that low-molecular-weight components of host plant extracts induce *vir* gene expression is at odds with the original report of Okker et al. (191) indicating that a protein was the inducer. At present, it is difficult to reconcile these apparently conflicting results.

Recognition and Attachment

Binding assays. Attachment of *A. tumefaciens* to a wound site is one of the first steps required for tumor formation. Binding data have been collected which contradict the observations that specific binding is a necessary prerequisite for tumor initiation (10, 153, 202, 265). Differences in assay techniques may be an explanation for the conflicting results. Individually, each assay has its merits, but the differences in the assay make them difficult to compare. Direct binding assays more accurately delineate the mechanisms and cell constituents necessary for attachment than do tumor inhibition assays. Tumor inhibition assays measure the effect of cell components on biological activity (i.e., inhibition of tumor formation by applied *Agrobacterium* spp.). Binding assays measure the adherence of *Agrobacterium* spp. to the plant surface irrespective of tumor formation. When binding assays are utilized, the biological activity of the *Agrobacterium* spp. should also be tested with a comparable biological assay.

The number of tumors in wounded pinto bean leaves and potato tubers is concentration dependent (10, 153, 202). An increase in numbers of bacteria inoculated causes an increase in the number of tumors. Development of a biological assay for tumor formation provides a method to study the initial interaction between host and *Agrobacterium* sp. Pretreatment of wounded pinto bean leaves with avirulent or heat-killed agrobacteria inhibits tumor formation by virulent strains (152). The outcome of the experiment is dependent upon the order of addition of avirulent and virulent strains, and upon time. The avirulent strains do not inhibit tumor formation if added 15 min after the virulent strains. These results suggest that discrete binding sites for *Agrobacterium* spp. exist on the plant surface.

Inhibition of tumor formation by avirulent strains has also been observed on potato (96) and Jerusalem artichoke (80) disks. The order of addition of the agrobacteria necessary for tumor inhibition implies that the first inoculated strain irreversibly occupies the available receptor sites; this indicates that bacterial attachment to a specific receptor site is required for crown gall tumor formation. Inhibition of tumor formation by avirulent bacteria is an indirect measurement of attachment.

These observations that avirulent strains inhibited tumor formation were extended to determine the cell surface components necessary for attachment. The effect of bacteria and plant cell wall components on inhibiting tumor formation by virulent strains was measured. The LPS of virulent and binding-proficient avirulent strains is an effective inhibitor of tumor formation on wounded pinto bean leaves (265). The LPS from *A. radiobacter*, a strain which does not bind to pinto bean, does not inhibit tumor formation. Separation of the O antigen from the lipid A portion of the LPS indicates that only the O antigen is inhibitory (18; M. H. Whatley, B. B. Lippincott, and J. A. Lippincott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, B8, p. 12). In contrast, in the potato tuber tumor assay, relatively high concentrations of LPS (2.5 μg/ml) from both virulent and avirulent strains of *A. tumefaciens* do not inhibit tumor formation (203). These discrepancies are difficult to reconcile but may be due to differences in the two bioassays. The LPS isolated from virulent *A. tumefaciens* inhibits the binding of agrobacteria to tobacco tissue culture cells (174). Once again, the LPS isolated from *A. radiobacter* does not inhibit binding of virulent *A. tumefaciens* to tobacco tissue culture cells. Both a direct (174) and an indirect (265) binding assay strongly suggest that the bacterial binding site for attachment contains LPS.

Evidence for the involvement of bacterial LPS in attachment to the plant surface has been obtained by using strains of *A. radiobacter* containing the Ti plasmid (185, 266). As mentioned previously, *A. radiobacter* strains are avirulent and nonbinding. The LPS of these strains does not inhibit tumor formation. Introduction of the Ti plasmid into *A. radiobacter* results in virulence. LPS from this strain does inhibit tumor formation by virulent *A. tumefaciens* (266). Curing of the Ti plasmid from some strains, however, does not result in the loss of the ability of LPS to inhibit tumor formation (266). These strains can still bind to carrot cells (167). Recently, it was reported that strains with plasmids pSa and Ti were avirulent and nonbinding. *Agrobacterium* spp. which lose plasmid pSa gain virulence, binding capabilities, and an LPS structure which inhibits tumor formation (185). These results suggest that the presence of plasmids can influence the adherence properties of *Agrobacterium* spp.

The nature of the plant cell wall receptor necessary for *Agrobacterium* attachment has not been elucidated, but it is

not synthesized in response to the bacterium. *Agrobacterium* cells bind to heat-killed or glutaraldehyde-fixed carrot cells as efficiently as they bind to living ones (171). The effect of isolated plant cell wall components on tumor formation has been examined. Pectin, PGA, and arabinogalactan inhibit tumor formation on wounded pinto bean leaves (209). PGA inhibits tumor formation at a concentration as low as 1 ng per leaf (209). Commercial pectin is approximately 10^4 times less effective in inhibiting tumor formation. Approximately 30% of commercial pectin contains methyl esterified carboxyl groups (155). The degree of methylation appears to determine whether or not the pectin fragment correlates with the level of tumor inhibition. Highly methylated forms are not inhibitory, but treatment with pectin methyl esterase results in tumor inhibition (209). Crude potato pectin and several PGA oligomers inhibit tumor formation on potato tubers (202). Contrary to previous results, however, the degree of methylation of potato pectin does not correlate with the level of tumor inhibition. Pectin does not inhibit adsorption of *Agrobacterium* spp. to potato tubers (204). Therefore, the effect on tumor formation seen earlier may not reflect effects on *Agrobacterium* binding. Isolated cell walls from monocots, crown gall tissue, or embryonic dicots do not inhibit tumor formation on pinto bean leaves (154). The list of plant cell wall components which do not inhibit tumor formation includes wheat germ, potato tuber, and kidney bean lectin, galactose, galactan, and glucuronic acid (155, 202, 205).

A compilation of the above observations suggests that the receptor on the plant surface is pectin and the bacterial receptor is LPS. The evidence for this suggestion, however, is equivocal and more research is needed. These cellular constituents may specifically interact with one another, resulting in a biological response, and therefore function as signal molecules. These initial interactions between the two surface receptors are ideal for the exchange of information. Upon interactions with the pectin portion of the plant cell wall, specific physiological responses may occur, perhaps mobilization of the T-DNA for transfer or induction of the *vir* genes. The effect of either of these two cellular constituents on gene expression needs to be assessed. The plant may respond to the LPS by inducing or repressing plant defense mechanisms or by preparing for transfer of the T-DNA.

Microfibril synthesis. Production of cellulose microfibrils by *A. tumefaciens* in response to attachment to carrot cell suspension cultures (72, 168, 171, 190) and protoplasts (172), or to tobacco leaf mesophyll or suspension culture cells and callus (116), has been reported. Cellulose microfibril production is not required for virulence (168). That is, mutants defective in cellulose synthesis remain virulent. Transposon mutants of *A. tumefaciens* defective in attachment (173) and cellulose synthesis have been isolated (168). The transposon mutations are located in the chromosomal DNA and the Ti plasmid. Washing of carrot cells with H_2O more readily removed *A. tumefaciens* cells which did not synthesize cellulose microfibrils. Synthesis of cellulose fibrils anchors *A. tumefaciens* to the cell wall and entraps other agrobacteria, forming large aggregates on the cell surface. *Agrobacterium* cells grown in the presence of soluble carrot extract develop cellulose fibrils. Avirulent agrobacteria defective in attachment synthesize cellulose fibrils at a lower rate than virulent strains (171). *Agrobacterium* attachment and fibril synthesis proceeds with heat-killed or glutaraldehyde-fixed carrot cells. The data suggest that interaction of *Agrobacterium* spp. with the plant cell receptor(s) induces the production of fibrils to further anchor the bacterium to the plant cell surface. Attachment generally occurs within 30 to 90 min, and the fibril production increases with time (72, 168, 171).

The number of fibrils formed by *A. tumefaciens* on tobacco is dependent on the type of tissue utilized (72). Strain A6, when attached to tobacco suspension culture cells, synthesizes greater numbers of cellulose fibrils than after its attachment to tobacco callus cells. A substantial number of fibrils are formed in response to attachment to tobacco callus cells; fewer fibrils are formed when strain A6 attached to tobacco mesophyll cells (72). This suggests that fibril synthesis is mediated by attachment to the plant cell and that plant cell type or culture conditions or both may affect microfibril synthesis.

β-2-Glucan. Mutants of *A. tumefaciens* with Tn*5* insertions in their chromosomal DNA have demonstrated that the chromosome encodes the functions necessary for attachment and virulence (80, 81). These chromosomal genes are distinct from those necessary for cellulose fibril synthesis (80). Attachment was assayed by the ability of the radiolabeled agrobacteria to adhere to tobacco tissue culture cells or freshly isolated *Zinnea* leaf mesophyll cells (80). Virulence was assayed by the ability to form tumors on *Kalanchoe* leaves (81). The attachment-defective mutants are unable to prevent tumor formation on Jerusalem artichoke slices by a virulent strain (80). Two loci are necessary for attachment; they have been designated *chvA* and *chvB* for chromosomal virulence (81). A low-molecular-weight β-2-glucan was isolated from hot phenol-water cell wall extracts of the virulent wild-type strain (207). The four Tn*5*-induced strains with mutations mapping in the *chvB* transcriptional unit do not synthesize β-2-linked glucan (207). The avirulent strains were complemented with a recombinant clone containing wild-type *Agrobacterium* DNA spanning the *chvB* region (81). Complementation restored the mutant's virulence and the ability to synthesize β-2-glucan (81, 207). The presence of a β-2-linked glucan secreted by *Agrobacterium* spp. has been reported by several investigators (115, 116, 276). The β-2-linked glucan is cyclic and ranges in size from DP = 17 to 30 D-glucosyl residues (74, 164, 174).

As mentioned above, β-glucans can be isolated from *Agrobacterium* spp. through procedures used for isolation of LPS. Previous results implicating LPS in attachment may be due rather to contaminating β-glucan. Some studies reporting a lack of LPS activity on *Agrobacterium* binding have used ultracentrifugation to purify the LPS (202). Such a purification step would result in the loss of β-glucan.

Results suggest that β-2-glucan plays a role in the attachment of *A. tumefaciens* to plant cells. β-Glucans have been implicated as signal molecules involved in the *Rhizobium*-legume symbiosis (1) and in the elicitation of phytoalexins (7, 57). The *Rhizobium* β-2-glucan enhances infection thread formation and nodulation (1). It is conceivable that the β-2-glucan isolated from *Agrobacterium* spp. could play a role analogous to the β-2-glucan isolated from *Rhizobium* spp. The *Agrobacterium* β-2-glucan is not the only component necessary for attachment to plant cell surfaces. Mutations in the *chvA* transcriptional unit do not affect β-2-glucan synthesis. What roles the β-2-glucans and the *chvA* gene products play in attachment and virulence have not been fully determined.

Specificity of oncogenesis. Monocotyledenous plants are generally not susceptible to crown gall disease (73). Lippincott and Lippincott (154) reported that the cell walls of monocotyledenous plants did not inhibit tumor formation on

TABLE 2. Signal molecules involved in *Agrobacterium*-crown gall disease

Identified signal molecule	Possible or unidentified signal molecule	Physiological response	Reference(s)
	Factor released in response to agrobacterial attachment	Microfibril synthesis	72, 168, 171, 190
	Plant extract (protein)	Induction of *virC* promoter	191
	Plant extract (phenolic compound)	Induction of *vir* promoter	234a, 234b, J. Chimera, T. J. Close, and C. I. Kado, unpublished data
Acetosyringone, hydroxyacetosyringone		Induction of *vir* promoter	234a
Opines		Conjugal transfer of Ti plasmid	118, 197
	β-1,2-Glucan	Attachment and/or virulence	207
	Factor produced by plant protoplasts	T-DNA circular intermediate	139

pinto bean leaves by virulent agrobacteria. These results initially suggested that *Agrobacterium* spp. could not bind to monocotyledenous plants, thereby rendering them resistant to crown gall. Binding of agrobacteria to monocots, however, has been reported (79, 82, 170, 190). The number of virulent *Agrobacterium* spp. bound to monocot cell suspension cultures is less than dicot cell suspension cultures (170, 190). Binding has been demonstrated in wheat, oats, corn, rice, and sorghum tissue cultures (170, 190). Mechanically isolated *Asparagus officinalis* cells are agglutinated by both virulent and avirulent strains of *A. tumefaciens* (82). This provides additional evidence that *Agrobacterium* spp. can bind to monocotyledenous plants. Recently, virulent wild-type *A. tumefaciens* strain A348 was shown to bind to cell suspension cultures of the monocot bamboo. Attachment of strain A348 to bamboo is indistinguishable from attachment to tobacco cell suspension cultures (79). Nonbinding avirulent mutants which did not bind to dicots (81) do not bind to bamboo (79). Washing of bamboo cells does not reduce the number of bound agrobacteria (79). This gives further support to the observation that *A. tumefaciens* can bind to monocots. These results indicate that monocot resistance to crown gall is not due to the inability of *A. tumefaciens* to bind to monocots.

Recently it was reported that the monocots *Chlorophytum capenae* and *Narcissus* cv. paperwhite can be transformed by *A. tumefaciens* (119). The integrated T-DNA is expressed in the monocots, since opines are detected in the wound site swellings. Opines are not detected in noninfected plant tissue or from wound sites inoculated with avirulent strains. T-DNA transfer is not accompanied by the formation of crown gall tumors. It has not been shown that the T-DNA-encoded *onc* genes are expressed. The range of monocots capable of receiving T-DNA needs to be examined. The *A. tumefaciens virC* promoter, fused to the *lacZ* gene, is induced by plant extracts from the monocot *Allium cepa*, but not *Z. mays* (corn) (191). Perhaps the ability to induce the *virC* promoter could be used as an assay for the potential for T-DNA transfer into monocotyledenous plants. The aforementioned results indicate that infection of monocotyledenous plants by *Agrobacterium* spp. is not based on a single genetic trait of either partner. Transformation of a monocot by the T-DNA of *Agrobacterium* spp. may be a multistep process requiring attachment and induction of genes necessary for virulence. The inability of *Agrobacterium* spp. to bind to the monocot or respond to a signal molecule necessary for induction of virulence genes could render the bacterium avirulent.

***Agrobacterium:* a passive or active partner in binding?** The necessity of the Ti plasmid for agrobacterium attachment to dicotyledenous plant tissue has been observed in several systems (174, 266). Curing of the *Agrobacterium* Ti plasmid, however, generally does not affect its ability to attach to tissue culture cells (80, 81, 169). The Ti plasmid-cured strain NT1 exhibits ambiguous binding properties to tissue culture cells. For all inoculum concentrations tested (10^3 to 10^7 cells per ml), NT1 binds to carrot cell suspensions to a level 25 to 40% that seen with the wild-type parent (167, 169). Less than 5% of strain NT1 binds to carrot suspension-cultured cells at a concentration of 10^3 cells per ml, but at a concentration of 10^7 cells per ml approximately 30% of the NT1 cell suspension bound to the carrot cells (167). Pretreatment of carrot cells with tissue culture media obtained from a carrot-NT1 cell suspension may have an effect on the ability of carrot cells to bind NT1. If the number of bacteria binding increases, this would suggest that a soluble substance is present in the media which enhances attachment.

It has been clearly established that attachment of agrobacteria to tissue culture cells is a physiological process independent of host cell viability (171). Killed agrobacteria, however, are incapable of attaching to tissue culture cells (168). Matthysse (167) reported that protein synthesis is not a prerequisite for attachment. This was concluded from the observation that attachment of the virulent wild-type strain A6 is not affected by the presence of tetracycline at a concentration effective in preventing [^{3}H]leucine incorporation into protein. From these data, it was concluded that, during the initial stages of attachment, the plant cell and the bacterium are passive partners (167). The effect of *Agrobacterium* and isolated cell wall components on host gene expression has not been assessed. The effect of fungal cell wall components, and the microorganism itself, on gene expression necessary for phytoalexin synthesis and disease resistance in suspension-cultured cells has been firmly established. Plant tissue culture provides an excellent tool to investigate the effect of agrobacteria on plant gene expression involved in the establishment of crown gall. *Agrobacterium* spp. may induce or repress the physiological processes necessary for the induction of crown gall disease. This is an area of needed research.

Concluding Remarks

The molecular events associated with the establishment of crown gall disease have not been clearly established. A list of identified and potential signal molecules involved in the *Agrobacterium*-crown gall disease is given in Table 2. The mechanisms involved in initiating crown gall disease are just beginning to be delineated. Preliminary data indicate that some of the *vir* gene products and circularization of the

T-DNA are induced by host plant extracts. Attachment of *Agrobacterium* spp. to tissue culture cells may be enhanced by a factor produced by the bacterium. All of these data suggest that there is an exchange of signal molecules between the host cell and *Agrobacterium* sp. The sequence of events and physiological responses associated with these signal molecules have not been delineated. This is an area of needed research which promises important results.

RHIZOBIUM-LEGUME SYMBIOSIS

The exchange of signal molecules between microbe and host is well exemplified by symbiotic relationships. The nitrogen-fixing *Rhizobium*-legume symbiosis has been studied extensively for almost a century. Even though the *Rhizobium*-legume symbiosis has received a great deal of attention, relatively little is known about the molecular mechanisms of the interaction. *Rhizobium* spp. only infect and nodulate a particular host. This specificity exhibited between *Rhizobium* spp. and their legume host is the basis of the following cross-inoculation groups: *Rhizobium leguminosarum*-pea, *R. phaseoli*-bean, *R. trifolii*-clover, *R. meliloti*-alfalfa, and *Bradyrhizobium*/*R. japonicum*-soybean. These cross-inoculation groups are a generalization rather than a specific corollary. The nodulating, nitrogen-fixing bacteria have been divided into two genera: the fast-growing *Rhizobium* and the slow-growing *Bradyrhizobium* species.

The establishment of the *Rhizobium*-legume nitrogen-fixing symbiosis is a complex developmental process. Infective rhizobia recognize and attach to host root hairs or root cells genetically predisposed to become root hairs. Host plant lectins have been suggested to mediate the specific recognition and binding of infective rhizobia to the root surface. *Rhizobium* cell surface polysaccharides have been shown to bind specifically to host plant lectins. Following attachment, infective rhizobia induce curling of the root hair by an unknown mechanism. The rhizobia become entrapped in the curl of the root hair, which appears important for initiation of the infection process. The host deposits new cell wall material internal to the point at which the rhizobia are entrapped by the curled root hair. The new cell wall material is used to form a tubular structure called the infection thread. Rhizobia are carried single file into the infection thread. The infection thread grows as it follows the host cell nucleus to the base of the root hair cell. Root cortical cells are induced to divide and differentiate into nodule tissue. The infection thread passes through the cell wall of the root hair and begins to branch into the adjacent, newly divided cortical cells. Rhizobia are released from the infection thread into the cytoplasm of cortical cells and are surrounded by a host-derived peribacteroid membrane. The rhizobia differentiate into bacteroids. Mature bacteroids are capable of reducing atmospheric nitrogen into ammonia which the plants assimilate for growth and development. Host-specific nodule proteins (leghemoglobin and nodulins) are also induced at an early stage of the infection process.

Establishing an effective nitrogen-fixing symbiotic relationship between the host legume and infective rhizobia requires cell-cell signal exchange. The factors exchanged in establishing the *Rhizobium*-legume symbiosis are not well characterized. The *Rhizobium*-legume symbiosis has been reviewed extensively (5, 14, 19, 66, 220, 257, 258).

Recognition, Attachment, and the Lectin Hypothesis

The molecular mechanisms of recognition in the *Rhizobium*-legume symbiosis has received much attention in the past decade. The current hypothesis receiving the most support is that host plant lectins interact selectively with compatible *Rhizobium* cell surface polysaccharides. Noninfective rhizobia, therefore, are thought not to interact with the host lectin. This hypothesis was first proposed by Albersheim and Anderson-Prouty (5) and is based on their work with elicitors and plant defense responses. They propose that recognition of a compatible *Rhizobium* sp. by the host lectin determines the outcome of the interaction. The amount of experimental evidence both for and against the lectin hypothesis is extensive. Due to the vast amount of literature pertaining to the involvement of host plant lectins in the recognition processes of the symbiosis, we cannot review all of the material here. We will discuss, however, the most important evidence, the latest information available, and the data indicating that lectin functions as a signal molecule. The original papers and numerous reviews should be consulted for more detail (5, 19, 66, 219).

Bohlool and Schmidt (34) were the first to provide experimental evidence demonstrating that lectins may function as recognition molecules or determinants of host specificity. They observed that fluorescein isothiocyanate (FITC)-labeled SBL bound specifically to 22 of 25 strains of *Bradyrhizobium japonicum* (34). The FITC-labeled SBL, however, did not bind to the 23 heterologous *Rhizobium* strains tested which do not nodulate soybean (34). This observation was subsequently confirmed (28) and expanded (25). *B. japonicum* strains which do not bind to the FITC-labeled SBL obtain lectin-binding capabilities after the cells are cultured in the presence of host root exudates (25). This indicates that the plant can induce changes in the *Rhizobium* cell surface which enables lectin binding to occur. Demonstration of specific binding of host lectins to compatible rhizobia was extended to several other legume-*Rhizobium* symbioses (63, 129, 195, 270).

Dazzo and colleagues have demonstrated a strong correlation between lectin binding and infectivity in the *R. trifolii*-clover symbiosis (63). Infective strains of *R. trifolii* contain polysaccharides which are antigenically cross-reactive with the clover root hair cell surface (63). This cross-reactive antigen binds to the purified clover lectin, trifoliin A (71). Trifoliin A has been shown to specifically interact with the LPS and capsular polysaccharide (CPS) of *R. trifolii* (65, 68). *R. trifolii* attachment to clover root hairs has been separated into two phases (66, 68). Phase I consists of *R. trifolii* attachment to the clover root lectin. The multivalent trifoliin A recognizes similar carbohydrate residues on *R. trifolii* and clover (63). The clover root lectin thus functions as a cross-bridge between *R. trifolii* and the root hair (63). For a comprehensive review of the *R. trifolii*-lectin interaction, see reference 66. The experimental data, however, do not conclusively prove that the lectin recognition hypothesis is correct. Most of the studies have used seed lectins rather than root lectins. Considerable evidence, however, has been reported for the specific interaction of root lectin with infective rhizobia. Clover root lectin has been isolated and shown to specifically interact with the cell surface polysaccharides of *R. trifolii* (61, 65, 71). Law and Strijdom (142) originally reported that the seed lectin of *Lotenonis bainesii* does not bind to the cell surface of its infective *Rhizobium* sp. The root lectin, however, does bind to the infective rhizobia (143). Root lectins which interact with their respective infective *Rhizobium* sp. have also been reported for soybean (232) and sweet clover (217). A pea root lectin (77, 120) has also been isolated, but it has not been determined if it interacts specifically with its symbiotic partner, *R. leguminosarum*.

Lectin-binding capabilities and optimal infectivity have been observed to be transient in several *Rhizobium*-legume interactions. Changes in *B. japonicum* infection and nodulation capabilities are directly correlated to the proportion of cells in a culture capable of binding to SBL (27). Lectin binding and infectivity are also related to culture age (27). A similar correlation is observed in the *R. trifolii*-clover symbiosis. The number of lectin receptors on the *R. trifolii* capsule at a particular culture age is directly correlated to the number of cells bound to clover root hairs (70, 227). The number of infection threads formed is optimal at the same culture age (121). Certain strains of *R. leguminosarum* exhibit similar transient lectin-binding capabilities (251). A correlation between optimal lectin binding to *R. leguminosarum* and infectivity needs to be established. Changes in *Rhizobium* lectin-binding abilities are due to alterations in the cell surface polysaccharides. After midexponential phase, *B. japonicum* strain USDA 138 cannot bind to SBL (28, 180). This change in lectin-binding ability is due to methylation of the CPS galactosyl residues and a reduction in encapsulation (180). The LPS of *R. trifolii* O403 binds to trifoliin A at a growth phase in which the LPS has increased its level of quinovosamine (121). Quinovosamine is the hapten of the LPS-trifoliin A agglutination reaction.

There are reports of inconsistencies in the interaction of lectins with *Rhizobium* spp. which weaken the lectin hypothesis. Several *Rhizobium* species bind to lectins isolated from plants which they do not infect or nodulate (64, 142, 217, 272). Furthermore, several legumes are infected and nodulated by more than one *Rhizobium* species, which defies the classical definition of cross-inoculation groups. For example, cowpea (*Vigna unguiculata*) is infected and nodulated by the cowpea miscelleny group (*Rhizobium* spp.) and by some *B. japonicum* strains (186). Another observation which has weakened the lectin hypothesis is that *Rhizobium* attachment to its host root is not specific (46, 201, 257a). Attachment is the relatively irreversible adhesion of microorganisms to surfaces. Specific attachment or a greater percentage of homologous rhizobia attaching to host roots has not been observed in several instances (46, 201, 257a). Lectin pretreatment of *B. japonicum* does not enhance its attachment to soybean roots (201). If lectin mediates attachment to the root, one would expect that lectin pretreatment of *B. japonicum* would enhance attachment.

Soybean lines which lack the 120,000-dalton seed lectin have been isolated (194, 206, 235). An insertion sequence blocks expression of the seed lectin gene in these lines (97, 259). These soybean varieties, however, are nodulated by several strains of *B. japonicum* (206). The existence of these seed lines is an obvious challenge to an essential role of lectin in nodulation. However, a second lectin present in the root at low levels has been detected (97, 218). A lectin has also been isolated from soybean roots by affinity chromatography, which removes galactose-binding proteins (246).

Carbohydrate residues (haptens) can specifically interfere with lectin-cell binding. Hapten inhibition of lectin binding to *Rhizobium* spp. has been reported in all investigations involving lectin-*Rhizobium* agglutination (28, 64, 120, 142, 143, 217, 272). Inhibition of *Rhizobium* binding to legume roots by the presence of lectin haptens has been reported for only a few *Rhizobium*-legume interactions. Hapten inhibition of *Rhizobium* binding to the root surface has been reported in soybean (232), pea (129, 130), and clover (65). Approximately 90% of the *R. trifolii* cells attached to clover root hairs can be removed by the trifoliin A hapten 2-deoxyglucose (65). Anomalous differing results have been reported in soybean. Vesper and Bauer (257a) reported that D-galactose inhibits attachment of *B. japonicum* to soybean root hairs but *N*-acetylgalactosamine does not. Both sugars are haptens for the SBL. Stacey and colleagues (232), however, observed by light and scanning electron microscopy that *N*-acetylgalactosamine and D-galactose inhibit binding of *B. japonicum* to *Glycine max* (soybean) and the wild ancestoral soybean (*G. soja*). These differences may be the result of using different methodologies. Stacey et al. (232) examined binding by microscopy, while Vesper and Bauer (257a) quanitated the rhizobia released from the root after ultrasonic vibration.

Host lectins functioning solely in the specific attachment of homologous rhizobia to the root does not appear to be the case. Dazzo and Truchet (66) recently reviewed the interactions between rhizobia and lectins, the phases of *R. trifolii* attachment to clover, and the factors which influence attachment. Attachment of *R. trifolii* to clover root hairs was separated into two distinct phases (66, 68). Phase I consists of *R. trifolii* attachment to the clover root lectin trifoliin A. Phase II involves adherence of *R. trifolii* to the root hair surface by extracellular microfibrils of unknown origin (66, 68). Extracellular microfibrils associated with the attachment of *R. trifolii* to clover have been reported previously (109). The identity and function of these extracellular microfibrils are unknown. Cellulose microfibrils are synthesized by *A. tumefaciens* in response to attachment to carrot cells (168). *Rhizobium* and *Agrobacterium* spp. are taxonomically related. *Rhizobium* sp. may synthesize cellulose microfibrils to firmly attach itself to the root surface. In fact, relevant data indicate that mutations in certain nodulation genes can affect production of extracellular microfibrils associated with the bacteria attached to clover root hairs (K. Smith and F. B. Dazzo, personal communication).

Pili are synthesized by both *B. japonicum* and *R. trifolii* in laboratory culture (S. Vesper and W. D. Bauer, submitted for publication). A reduction in *B. japonicum* attachment (90%) and nodulation (80%) occurs when cell suspensions are treated with either crude antiserum or purified immunoglobulin G against isolated pili. The amount of antiserum or immunoglobulin G necessary to achieve this inhibition, however, appears to be unusually high. Nevertheless, these data suggest that pili may be involved in the adherence of *B. japonicum* to soybean roots. Only a small percentage (1 to 16%) of the *B. japonicum* cell population was piliated (Vesper and Bauer, submitted). The percentage of piliated *Bradyrhizobium* spp. when cultured in the presence of host roots or root exudates would indicate whether piliation increases as a response to a host plant.

Adhesion (attachment) is the relatively stable irreversible attachment of bacteria to surfaces. The role adhesion has in establishing an effective *Rhizobium*-legume symbiosis has not been completely determined. Firm attachment of infective *R. meliloti* strains to the host root enhances competitive ability (255). Strains which adhere loosely to the root surface are generally less competitive (255). Adhesion may be important for the exchange of signal molecules between the host and bacterium. The initial contact of a microbe with the plant/root surface may be a random event. Adsorption which would follow contact is probably dependent upon general surface properties of a host and microbe. Adsorption is most likely to be a reversible phase (i.e., phase I attachment of *R. trifolii* [66, 68]). Synthesis of extracellular adhesins would enable the *Rhizobium* sp. to tightly bind to the plant surface. *Rhizobium* adhesins could be extracellular cellulose microfibrils (109, 168) or pili (Vesper and Bauer, submitted)

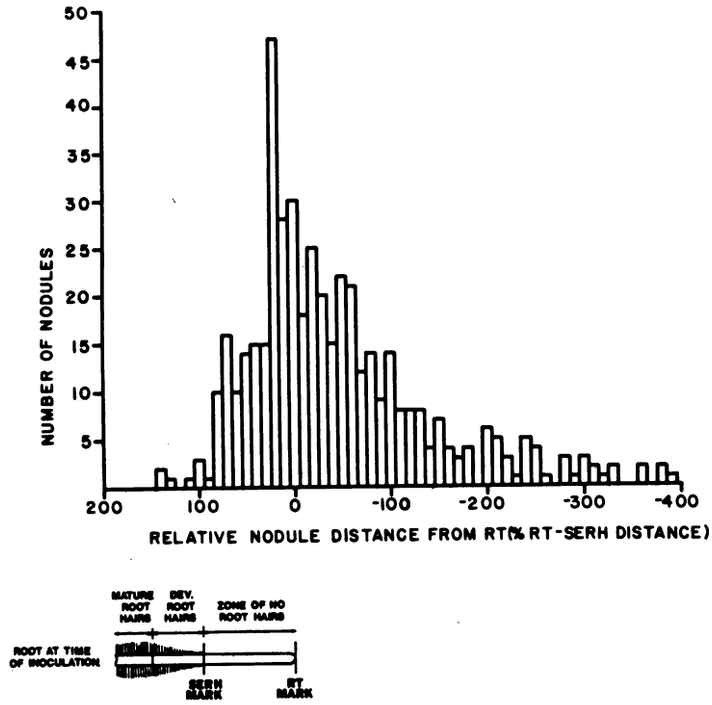

FIG. 3. Diagrammatic representation of a soybean root at the time of inoculation and profile of the distribution of nodules formed by wild-type *B. japonicum* strain USDA 110. To demark the zone most susceptible to infection and nodulation, the RT and SERH were marked on the surface of the growth pouch at the time of inoculation. Seedlings were inoculated with 1.0 ml of a 10^8-cells per ml suspension of *B. japonicum* strain USDA 110 grown to midlog phase in YEM medium (101, 103). Nodule location was scored 14 days postinoculation. The profile of the distribution of nodules formed on soybean inoculated with *B. japonicum* strain USDA 110 was constructed after measuring the distance of all primary root nodules relative to the RT mark to the nearest 0.1 mm. The relative distance of each nodule on the primary root from the RT mark was calculated as a percentage of the RT-SERH distance of a given plant (30, 101).

or both. *Rhizobium* cell surface polysaccharides or proteinaceous membrane receptors or both could then recognize and bind to surface receptors. Recognition of certain molecules on the cell surface could signal or induce physiological responses which aid development of the symbiosis. The correlations between lectin binding and *Rhizobium* infectivity are too strong to disregard as coincidental. Until recently the effect of *Rhizobium*-lectin interactions on infectivity had not been investigated. Data are beginning to accumulate which demonstrate that lectin binding induces physiological changes in rhizobia and that this interaction is specifically induced by host lectins. The recognition of signal molecules (such as lectin) during the adhesion process would facilitate initiation of the infection process. Adhesion could also enable host cell wall polysaccharide-degrading enzymes to modify the *Rhizobium* cell surface. The modified *Rhizobium* cell surface polysaccharides could induce host responses necessary for infection.

Location and Regulation of Infectible Host Cells

Identification of the infectible host cells on the legume root has enabled development of a bioassay to investigate the recognition processes involved in establishing the symbiosis (22–24, 26, 30, 101, 103). Bhuvaneswari and colleagues (26, 30) demonstrated the developmental restriction of nodulation on the roots of soybean and cowpea in the area between the root tip (RT) and smallest emerging root hair (SERH). Seedlings were grown in clear plastic growth pouches enabling one to mark the RT and SERH at the time of inoculation. Figure 3 is a diagramatic representation of the root at the time of inoculation and the subsequent nodulation profile of soybean inoculated with the wild-type *B. japonicum* strain USDA 110. Nodulation is scored relatively to the RT mark made at the time of inoculation. Infection (41, 200) and nodulation (26, 30, 101) fail to occur in the region of fully elongated (mature) root hairs present at the time of inoculation. Infection and nodulation occur occasionally in the region of developing root hairs. The area between the RT and SERH, the no-root-hair zone, is where the majority of infections (41, 200) leading to subsequent nodulation occur (26, 30, 101). These root cells become progressively less susceptible to nodulation as they differentiate into mature root hairs. Acropetal development of the root allows the root cells to be susceptible to infection (41) and nodulation (30) for a period of only 4 to 6 h. This time limit for nodule initiation within the RT-SERH zone enables one to infer the relative rate of infection by the position of the uppermost nodule from the RT mark.

The infectible root cells of soybean and cowpea are not typical of all legumes. Mature, developing, and no-root-hair zones of white clover are equally susceptible to infection by *R. trifolii* (26). Alfalfa exhibits a nodulation pattern similar to that of white clover, but the frequency of nodulation in the mature and developing regions is lower (26). This lower susceptibility in mature and developing root hairs exhibited by alfalfa may be due to a decreased susceptibility in infection or a lower proportion of successful infections. In

soybean and cowpea it has already been demonstrated that the mature and developing root hair regions are less susceptible to infection by rhizobia (41, 200).

High levels of nodulation are not sustained on the primary root of soybean as the root develops in the presence of rhizobia (Fig. 3) (30, 101). In regions of the root 10 to 15 h younger than the region most susceptible to nodulation at the time of inoculation, nodulation frequency decreases to 20% of the maximum (198). This suggests that either a host regulatory mechanism prevents excessive nodulation of the root or the number of viable rhizobia capable of nodulation is to low to support the higher levels of nodulation. Exposure of the root to a second inoculum of *B. japonicum* 15 h after the original inoculum does not increase the frequency of nodulation in the younger regions of the root (198). These data indicate that the host has a rapid regulatory response which inhibits subsequent nodulation of younger root tissue. Inhibition of subsequent nodule formation in younger regions of the root is apparently caused by termination of the infection process. Anatomical studies have shown that the younger regions of the root are as susceptible to infection as the region between the RT and SERH at inoculation (41). The host regulatory response apparently inhibits nodule emergence (41). Inhibition of subsequent nodulation by the host is specifically induced by infection of homologous rhizobia (198). If the original inoculum is *R. trifolii*, *R. leguminosarum*, or H_2O and the second inoculum is *B. japonicum*, the younger region of the root exhibits a frequency of nodulation comparable to that induced by inoculation with *B. japonicum* alone (198). UV-killed *B. japonicum* does not elicit inhibition of nodulation of the younger region of the root (198). Inhibition of soybean nodulation has also been demonstrated in the split root system (138). Inoculation of one side of a split root results in inhibition of nodulation of the other side of the split root within 96 h (138). Nodule expression is not related to the appearance of nodules or nitrogenase activity on the side inoculated with *B. japonicum* at time zero (138). This demonstrates that regulation of nodulation is not restricted to cells in direct contact with the inoculated cells (138). The results indicate that the host can regulate the degree of nodulation by monitoring the number of *Rhizobium* infections. Apparently, a diffusible factor must be produced that can travel from one side of the split root to the other.

The host signal molecule, induced by the homologous rhizobia, which inhibits nodule emergence has not been determined. It is conceivable that the signal is one of the nodulin proteins induced during infection and nodulation. Inhibition of nodule emergence (188), however, appears to occur prior to the appearance of nodulin mRNA (91). The availability of combined nitrogen is another mechanism whereby the host legume inhibits nodulation. Most infections abort before they develop into a nodule (41, 182, 189, 200). The availability of exogenous nitrogen can reduce the number of infection threads initiated and increase the percentage of aborted infection threads (182, 183). Exogenous nitrogen can also induce premature senescence of effective nodules. The data indicate that nodule emergence and maintenance are dependent upon an internal regulatory mechanism that exists prior and subsequent to nodulation and nitrogen fixation. Low concentrations of nitrate have been shown to increase nodule number and nodule fresh weight (177). Higher concentrations of nitrate (>5 mM) inhibit nodulation and nodule fresh weight (177).

Recently, a nitrate-tolerant and supernodulating soybean mutant (nitrate-tolerant symbiotic mutant [*nts*]) was isolated (43, 176). Germinated soybean seeds were mutagenized with ethyl methanesulfonate and planted, and mutant seeds were isolated which nodulated in the presence of exogenous nitrogen (43). At a concentration of 5.0 mM KNO_3 the *nts* mutants exhibited a 3.7- to 38-fold increase in nodule number over the wild-type soybean cultivar (43). Nodule fresh weight of the *nts* mutants is also greater than that of the wild type cultured on 5 mM KNO_3 (43). In the absence of nitrogen, nodule number and nodule fresh weight of the *nts* mutants are also greater than those of the wild type (43, 176). Evidently, nodulation of the *nts* mutant is limited by the space available for nodule formation. Both the wild type and the *nts* mutant 382 have similar nitrate reductase activity. This indicates that *nts* mutant 382 is not defective in nitrate assimilation. The mutated gene could possibly be the same gene that regulates the depression of nodule development in soybean (30, 138, 198, 200). Isolation of the *nts* mutant could provide an excellent tool to investigate the genes involved in regulating infection and subsequent nodulation. Comparison of the nodulin mRNAs and translation products of the wild type and *nts* mutant would be helpful. Further characterization of the *nts* gene and its control of nodulation could help to increase the nitrogen content of legumes.

Induction of Gene Expression by the Presence of the Host

The nodulation of cowpea and soybean is developmentally restricted to the area between the RT and SERH. This area, marked at the time of inoculation, remains susceptible to infection and nodulation for approximately 6 h, due to the acropetal development of the root (30). The location of the uppermost nodule relative to the RT mark enables one to infer the relative rate at which nodulation is initiated. Several *Bradyrhizobium* strains have been found to exhibit a delay in the initiation of nodulation (22, 101). That is, nodules appear well below the RT mark made at the time of inoculation. The wild-type *Bradyrhizobium* strain generally nodulates in an area near the RT mark (Fig. 3) (22, 26, 30, 101). The delay in nodule initiation can be phenotypically reversed by pretreating the *Bradyrhizobium* stains in host root exudates (22, 101, 103). This indicates that the root excretes substances which induce physiological processes necessary for nodulation. Two independent laboratories have been investigating these observations. Bhagwat and Thomas (22–24) have been investigating the physiological effect cowpea root extracts have on *Bradyrhizobium* sp. strains 32H1 and 1001. Our laboratory (101–103a) is examining the plant product which initiates this physiological response and the effect it has on *B. japonicum*.

Bradyrhizobium sp. strain 32H1 normally initiates nodulation in cowpea within 7 h after inoculation (22). Preincubation of strain 32H1 in cowpea root exudates for 4 to 6 h prior to inoculation of cowpea seedlings results in nodulation initiation within 2 h (22). Characterization of the cowpea root exudate indicates that the active component is insensitive to heating, but the exudate has not been characterized further (22). Furthermore, stationary-phase cultures of *Bradyrhizobium* sp. strain 1001 initiate a delayed nodulation response similar to strain 32H1. Midexponential-phase cultures of strain 1001 initiate a prompt nodulation response on cowpea (23, 24). Stationary-phase cultures of strain 1001 can initiate a nodulation response like midlog phase cultures, if the stationary-phase cultures are preincubated in cowpea root exudates for 4 to 16 h prior to inoculation (23, 24). Cowpea root exudates induce changes in the CPS of stationary-phase cultures of strain 1001. Cellulose acetate membrane electro-

phoresis and diethylaminoethyl-Sephadex chromatography of purified CPS of log-phase cultures of strain 1001 demonstrate the presence of two bands/peaks in the native CPS and three bands/peaks in root exudate-treated CPS (24). The sugar composition of the purified CPS changes following incubation of strain 1001 in cowpea root exudates (24). Root exudate-treated CPS shows an increase in the content of galactose and a decrease in mannose content (24). The higher level of mannose reported for the untreated control, however, may represent mannan contamination from yeast extract in the growth medium. The sugars arabinose and xylose also appear in the CPS isolated from root exudate-treated cells of strain 1001 (24). Treatment of seedlings with 2 μg of CPS (from root exudate-treated cells) prior to inoculation of untreated cells results in enhanced nodulation above the RT mark. CPS from untreated cells or fractionated CPS from treated cells does not enhance nodulation above the RT mark (24). This suggests that the component(s) in cowpea root exudate (22) induces a structural modification of the CPS of *Bradyrhizobium* sp. strain 1001. Due to the complex nature of root exudates it cannot be assumed that the factor(s) found in cowpea root exudate induces modification of strain 1001 CPS. The changes seen could be due to the nutritional environment of the root exudate. Isolation and purification of the cowpea root factor(s) and its effect on strain 1001 CPS nodulation-enhancing characteristics have to be determined before any correlations can be made. Interaction of the modified CPS with the cowpea root surface appears to signal the host that a compatible microsymbiont is present for infection and nodulation. Capsule synthesis is specifically induced in *Bradyrhizobium* sp. strain 1001 by cowpea and mung bean root exudates (23). Strain 1001 effectively nodulates both cowpea (*V. unguiculata*) and mung bean (*V. radiata* [23]). Root exudates obtained from *Trigonella toenum-graecum* and *Medicago sativa* do not enhance capsule synthesis in strain 1001. Infection of clover by *R. trifolii* is enhanced by pretreatment of the clover seedings with *R. trifolii* surface polysaccharides. This enhancement is discussed below.

Cowpea root exudate contains a factor(s) which enhances the nodulation of *Bradyrhizobium* sp. strain 32H1 (22). Root exudates obtained from cowpea plants grown in the presence of 5 mM NH_4Cl are not capable of inducing a faster nodulation response (22, 24). Immunologically detectable levels of the clover root lectin trifoliin A on the root surface decreases as the concentration of NO_3^- or NH_4^+ is increased (62). In addition, lectin released from plants treated with NO_3 possesses <5% of the activity of that from untreated plants (226). It is conceivable that the component(s) in cowpea root exudate which enhances the rate of nodule initiation is also a lectin and that its expression or activity is inhibited by the presence of exogenous nitrogen.

B. japonicum mutant strain HS111, derived from strain USDA 110, exhibits a slow-to-nodulate mutant phenotype (233). Nodules do not appear until 20 to 22 days after inoculation versus 10 to 12 days for the wild-type strain USDA 110 (101, 233). Mutant strain HS111 nodulates well below the RT mark, with the average distance of the uppermost nodule being approximately 18 mm below the RT mark made at the time of inoculation (101–103a). Mutant strain HS111 is apparently defective in the initiation of nodulation and in the rate of nodule development (101). The strain HS111 mutant phenotype can be phenotypically reversed by preincubation in soybean root exudates, SBL, or a galactose-specific soybean root lectin (101–103a).

SBL mimics the effect of root exudate pretreatment of mutant strain HS111 (102, 103). Pretreatment of mutant strain HS111 with SBL or root exudate results in similar relative rates of infection as in the wild type (102, 103) and shows similar time courses for maximum enhancement (102). The presence of the SBL hapten D-galactose also inhibits nodulation enhancement by SBL or root exudate (103). Removal of galactose-specific lectins from soybean root exudates by affinity chromatography results in the inability of the root exudate to enhance the nodulation characteristics of strain HS111 (103). Lectin eluted from the affinity column by D-galactose and then dialyzed to remove the galactose did induce nodulation competence in mutant strain HS111.

Soybean lines have been isolated which lack the 120,000-dalton seed lectin *le le* (97, 194, 206, 235). The soybean cultivar T102 lacks seed lectin expression, due to a genetic insertion in the SBL structural gene (97, 259). This soybean line is nodulated by *B. japonicum* (206). Root exudates obtained from cultivar T102 induce nodulation competence in mutant strain HS111 (103). mRNA isolated from soybean roots, when translated in vitro, produces a protein immunologically cross-reactive with the seed lectin (218). A galactose-specific seed lectin has been isolated from five soybean cultivars which were previously considered to be lectin negative (246). This seed lectin was isolated by affinity chromatography of defatted *le le* seeds and found to be present in a concentration 1,000- to 10,000-fold less than SBL-positive cultivars (246). A lectin has been purified from soybean roots which exhibits the same sugar specificity, and has nearly identical molecular weight, electrophoretic patterns, and amino acid composition, as the seed lectin (92). This indicates that the galactose-specific root lectin and the seed lectin are genetically distinct, but share a common affinity for galactose and induce the same physiological response in *B. japonicum*.

Nodulation competence is induced in *B. japonicum* strain HS111 specifically by the galactose-specific SBL. Root exudates and galactose-specific lectins obtained from other legumes and nonlegumes are incapable of inducing nodulation competence in *B. japonicum* mutant strain HS111 (103a). The physiological effect of lectin on the wild-type *B. japonicum* strain USDA 110 was demonstrated by two independent approaches. The first approach pretreated the wild type with root exudate or lectin for 72 h, inoculated the seedlings, and then scored the plants for nodule number 6 weeks later (103a). Both the root exudate and lectin pretreatments increase nodule numbers formed by the wild type approximately 2.5-fold compared with the untreated control. *B. japonicum*, at a concentration of 10^4 cells per seedlings, exhibits a delay in nodule initiation similar to mutant strain HS111 (27, 103a). This suggests that only a small percentage of the wild-type cell population is physiologically capable of initiating nodulation at the time of inoculation. A greater percentage of the wild-type population is capable of initiating a prompt nodulation response following lectin or root exudate pretreatment (103a). That is, the wild-type cells do not exhibit a delay in the initiation of nodulation after pretreatment with SBL or root exudate. A direct correlation has been established between the percentage of plants nodulating above the RT mark and the percentage of cells binding to soybean lectin (27). The data indicate that lectin also induces nodulation competence in the wild-type *B. japonicum*.

The ability of lectin or root exudate to affect nodulation by *B. japonicum* is prevented in the presence of rifampin or chloramphenicol (103a).

Apparently, lectin functions as a signal molecule which induces the expression of genes necessary for nodulation. Lectin may be involved in inducing infection thread formation or in infection thread maintenance. It is conceivable that soybean lectin alters the CPS of *B. japonicum* in a manner analogous to the alterations of *Bradyrhizobium* sp. strain 1001 CPS by cowpea root exudate. The effect of lectin on the cell surface polysaccharides of *B. japonicum* needs to be assessed.

Lectin pretreatment does not enhance attachment of the mutant strain HS111 to the soybean root as determined by the method of Pueppke (201). Furthermore, root hair curling (Hac) is expressed at wild-type levels in mutant strains HS111 with or without lectin pretreatment. The effect of lectin on infection thread formation has not been examined.

Promoters of *R. fredii* fused to the *lacZ* gene are induced by soybean and kidney bean root extracts (192). Root extracts obtained from other legumes or nonlegumes do not increase β-galactosidase levels (192). Characterization of the active factor(s) which induces promoter activity has not been reported. More recent work suggests that the inducer in the root exudate is a low-molecular-weight organic acid (M. J. Sadowsky, personal communication). Genes of *R. meliloti* important for early nodulation function are expressed only in the presence of plant exudates (181). The *nodC* gene fused to *lacZ* is expressed only in cultures exposed to alfalfa plant exudates (181). A low-molecular-weight, heat-stable factor present in the alfalfa exudates is responsible for this gene induction (181). It is presently unclear whether the low-molecular-weight factor from soybean is similar to that obtained from alfalfa. These data are similar to the observation that *B. japonicum* gene expression necessary for nodulation competence is specifically induced by soybean root exudate or soybean lectin (103a). A similar physiological response may be induced by several different host products.

Host-Synthesized Polysaccharide-Degrading Enzymes

Rhizobial polysaccharide-degrading enzymes from legume roots have been identified (69, 230). Clover roots (*Trifolium repens*) release polysaccharide-degrading enzymes which alter the capsule of *R. trifolii* (69). Incubation of heat-fixed encapsulated cells of *R. trifolii* in clover root exudate for 1 to 2 h results in trifoliin A binding uniformly around the cell surface (69). Continued incubation of *R. trifolii* in clover root exudates for an additional 4 to 8 h results in polar attachment of trifoliin A to one end of the cell (69). These results suggest that the capsule of *R. trifolii* is degraded by the enzymes in the root exudate. *R. trifolii* then synthesizes new capsule material which is preferentially deposited on one pole of the cell (69). Polar attachment of FITC-labeled SBL to the cell surface of *B. japonicum* has also been reported (35). Transmission electron microscopy shows that the capsule and lectin-binding properties of *B. japonicum* USDA 110 are located at one pole of the cell (256). The lectin-binding sites of *B. japonicum* strain USDA 138 have been shown to be oriented throughout a complete capsule which surrounds the cell (40). The orientation of the capsule and location of lectin-binding sites should be observed in cells which have been cultured in the presence of the root or root extracts.

Root enzymes isolated from root extracts of *Trifolium repens* partially degrade all polysaccharides obtained from its symbiont *R. trifolii*. The *Rhizobium* surface polysaccharides that have been examined include the exopolysaccharide (EPS), CPS, LPS, and a low-molecular-weight polysaccharide (230). The polysaccharides isolated from *R. leguminosarum* are not degraded as extensively by the *Trifolium repens* root extract enzyme preparation (230). Conversely, the *Pisum sativum* root extract enzyme preparation more effectively degrades its symbiotic partner's polysaccharides (*R. leguminosarum*) than the heterologous *R. trifolii* polysaccharides (230). These observations suggest that the host preferentially degrades the cell surface polysaccharides of its symbiotic partner. The question arises whether the newly synthesized cell surface polysaccharides are structurally the same. As stated previously, *Rhizobium* lectin-binding sites are dependent upon the growth stage of the cell (27, 28, 65, 121). Strains of *B. japonicum* which do not bind FITC-labeled SBL can do so at very low levels following preincubation with soybean root exudates (25). Host-derived polysaccharide-degrading enzymes may specifically degrade their symbiotic partners' cell surface to promote or facilitate lectin binding or root adherence. Polar attachment of rhizobia to legume roots has been observed by numerous investigators (61, 65, 68, 215, 247). The effect of polysaccharide-degrading enzymes released from clover roots on the capsule of *R. trifolii*, trifoliin A binding, and polar attachment has been reported (68, 215). Host-derived polysaccharide-degrading enzymes apparently play an important role in the initial stages of the infection process.

Biological Activity Induced by *Rhizobium* Surface Polysaccharides

Interaction of the surface polysaccharides of *Rhizobium* spp. with the host cell surface undoubtedly plays a role in the establishment of the symbiotic state. Which surface polysaccharides are involved, their biological functions, and how they regulate their biological activity are just beginning to be determined. *Rhizobium* species produce a variety of cell surface polysaccharides which exhibit biological activity. These include the EPS, CPS, LPS, and β-2-glucans.

Root hair curling. One of the first visible steps in the infection process of legumes by compatible rhizobia is the curling of root hairs (hair curling, Hac; 258). Little is known about the mechanisms of root hair curling. Bauer (19) discusses theoretical mechanisms of root hair curling, and that review should be consulted for more detailed information. Cell surface components which have been reported to cause root hair curling will be discussed. The first indication that root hair curling can be induced by a signal molecule is from the observation that *Rhizobium* culture filtrates induce root hair curling (122, 231, 273, 274).

Yao and Vincent (273) showed that marked root hair curling occurred only when seedlings were inoculated with the viable homologous cells. Heterologous rhizobia induce moderate curling and branching. *Rhizobium* sp. strain 127E15 is Nod^+ Fix^+ on *Phaseolus lunatus* and Nod^+ Fix^+ on *Phaseolus vulgaris* (222). *R. phaseoli* strain 127 K14, however, is Nod^+ Fix^+ on *Phaseolus vulgaris* and Nod^- Hac^- on *Phaseolus lunatus* (222). When *Rhizobium* sp. strain 127E15 nodulates a host of a different cross-inoculation group, it elicits the same specific host responses as it does from a host of the same cross-inoculation group.

Several researchers have attempted to characterize the active component(s) responsible for root hair curling. Cell-free culture filtrates, EPS, CPS, and extracellular protein fractions have all been implicated as possible root hair curling factors (81, 122, 231, 273, 274). Isolated fractions,

however, generally do not cause marked root hair curling as do viable cells (86). Isolation and purification of cell surface components may result in modifications of the active factor(s). Ervin and Hubbell (86) suggest that two fractions, both containing polysaccharide and one also containing protein, are necessary for nonattenuated root hair curling. This suggets that two or more signal molecules are necessary for marked root hair curling. Culture filtrates of *R. trifolii* grown in the presence of white clover exhibit greater hair curling activity than culture filtrates of *R. trifolii* grown on laboratory media (29, 231). The activity of root media alone on root hair curling was not reported (29). A heat-stable factor(s) and a heat-labile factor were reported to be necessary for marked curling by culture filtrates of rhizobia grown in the presence of roots (29, 231, 274). The *Rhizobium* cell surface may be modified by polysaccharide-degrading enzymes from the root (69, 230), which results in the synthesis of a biologically active hair curling factor or a modification of the cell surface to produce a biologically active factor. The heat-stable dialyzable factor has biological activity (29, 274). The heat-labile nondialyzable factor(s) may be enzymes which modify the *Rhizobium* cell surface since the factor does not cause root hair curling by itself (227).

Root hair curling genes. Transposon Tn*5*-induced mutants of *R. trifolii*, *R. meliloti*, *R. leguminosarum*, and *Rhizobium* sp. strain ANU250, defective in root hair curling (Hac) and nodulation (Nod), have been isolated (78). These mutations mapped on the symbiotic (Sym) plasmid of each strain. Transfer of heterologous Sym plasmids into the mutant strains results in effective root hair curling and nodulation abilities on their original hosts (78). Therefore, *hac* gene function is not species specific. Also the data indicate that the genes necessary for root hair curling are functionally conserved in a variety of *Rhizobium* species. *Rhizobium* EPS, CPS, and an extracellular protein fraction have been implicated as possible root hair curling factors (2, 122, 231, 273, 274). *R. trifolii* mutants defective in root hair curling (Hac) produce an EPS which is compositionally identical to the wild-type EPS (42a). Other work, however, focusing on quantitative differences in noncarbohydrate substituents (acetate, pyruvate, and β-hydroxybutyrate) utilizing ^{1}H-nuclear magnetic resonance indicate that the CPS of these same mutants are altered (A. E. Gardiol, R. I. Hollingsworth, and F. B. Dazzo, personal communication). The results do not exclude the possibility that the *hac* genes are induced by the host and that the *hac* gene product(s) alters the structure of the EPS or CPS or both into biologically active structures. Second, the *hac* gene product could possibly encode the protein factor (273) hypothesized to be involved in root hair curling. The effect of the purified *hac* gene product(s) on root hair curling itself or on the structure of the EPS or CPS or both should be examined.

Expression of the *hac* genes is apparently controlled by the presence of the plant. These genes are not expressed in free-living rhizobia or in the bacteroid form. In the presence of alfalfa exudates, however, Mulligan and Long (181) have demonstrated the expression of *nodC*:*lacZ* fusions. The factor(s) in the exudates essential for the induction of this *hac* gene is of low molecular weight and heat stable (181). A second *hac* gene, *nodD*, is necessary for the induction of *nodC* and is likely a regulatory gene. Innes et al. (122a) have also reported the induction of *nod* gene expression in *R. trifolii* by a low-molecular-weight component in root exudates. This factor has recently been identified as luteolin, a flavone (S. Long and B. G. Rolfe, personal communications). This compound is normally synthesized by plants, using much of the same pathway used to produce flavonoid-derived phytoalexins (Fig. 2).

Increased infection and nodulation. Pretreatment of clover (1, 2, 67, 110) and cowpea (24) seedlings with low concentrations of *Rhizobium* cell surface polysaccharide prior to inoculation results in increased infectivity and nodulation. Cyclic β-2-glucans, EPS oligosaccharide fragments, CPS, and LPS have all been implicated as mediators of increased infection and nodulation (1, 2, 24, 67). We have discussed the enhanced rate of infectivity of a stationary-phase culture of *Bradyrhizobium* sp. strain 1001 following incubation in cowpea root exudate. Cowpea root exudate apparently has an effect on the composition of the CPS of *Bradyrhizobium* sp. strain 1001 (24). Pretreatment of cowpea seedlings with as little as 2 μg of the host-modified CPS results in enhanced infectivity of untreated stationary-phase cells of strain 1001 (24).

Increases in infection thread formation and nodule number have been reported following treatment of the roots with trifoliin A-binding oligosaccharide fragments from the CPS and EPS of *R. trifolii* strain O403 (2). The oligosaccharide fragments were synthesized by treating the isolated *R. trifolii* CPS and EPS with acidic heteropolysaccharide lyases obtained from lysates of two *R. trifolii* bacteriophages (117). Pretreatment of white clover seedlings with as little as 2.5 μg of the CPS or EPS oligosaccharide fragment prior to inoculation with wild-type *R. trifolii* results in a concomitant 70 to 106% increase in infection threads (2). Higher concentrations are not as effective in promoting infection thread formation (2). The EPS oligosaccharide fragments increase infection thread formation, while the EPS does not. Both the CPS polysaccharide and CPS oligosaccharide fragments increases infection thread number (2). This suggests that the CPS fragment is the naturally occuring polysaccharide involved in the infection process. Depolymerization kinetics and carbohydrate substitution studies of the CPS and EPS oligosaccharide products of the acidic heteropolysaccharide lyases indicate that the two polymers are different (117). The biologically active form of *R. trifolii* cell surface CPS, LPS, or oligosaccharide fragments (EPS) bind to trifoliin A (1).

Neutral cyclic β-2-glucans of low molecular weights are synthesized constitutively by both *Agrobacterium* and *Rhizobium* spp. (1, 56, 74, 115, 116, 207, 276). Abe and colleagues (1) demonstrated that β-2-glucan of *R. trifolii* promotes infection and nodulation in white clover seedlings. As mentioned previously, a cyclic β-2-glucan is missing in an avirulent, attachment-defective mutant of *A. tumefaciens* (207). Virulence, attachment, and the cyclic β-2-glucan are regained when the mutant strain is complemented with wild-type DNA homologous to that region (207). Cyclic β-2-linked glucans may serve as signal molecules from both *Agrobacterium* and *Rhizobium* spp. Host cell interaction with the cyclic β-2-glucans may induce physiological responses within the host which assist in establishing an infection. Correlation between the ability of *Rhizobium* spp. to synthesize β-2-glucans and the ability to infect or the efficiency of infection of the host needs to be assessed. *B. japonicum* strain 3I1b71a produces two extracellular, 3-linked, 6-linked, and 3,6-linked β-D-glucans (83). The extracellular 3,6-linked β-glucan of *P. megasperma* f.sp. *sojae* is capable of eliciting phytoalexin synthesis in soybean (15, 19). This raises the question of how rhizobia can suppress the host's defense response upon infection if at the same time the rhizobia have cell surface components capable of eliciting defense responses. Control of the plant's induced defense responses may be a key to understanding the main-

tenance and stability of the infection process by rhizobia. The nodule tissue of a Fix$^-$ strain (61-A-24) of *B. japonicum* has a 10-fold-higher level of phytoalexin (glyceolin I) than nodule tissue formed by Fix$^+$ strains (61A101 and USDA 110 [263]). Glyceollin I accumulates in nodule tissue, formed by the Fix$^-$ strain 61-A-24, only after the peribacteroid membrane disappears (264). This suggests that the peribacteroid membrane plays an important role in preventing the induction of the host's defense mechanisms.

The observation that lectin-binding cell surface polysaccharides enhance infection by rhizobia has several implications. This suggests that interaction of rhizobia with a host lectin could result in the induction of specific physiological responses in both the host and the rhizobia. Rhizobia respond by inducing gene expression necessary for nodulation; the host responds to the lectin-binding polysaccharides by preparing the host for infection. The rhizobia can therefore initiate and develop an infection thread more rapidly and progress to a point where the host can no longer effectively regulate the number of infections. The final physiological result is an increase in the efficiency of infection and nodulation. In addition, the fact that the biologically active cell surface oligosaccharides have lectin-binding capabilities may be just coincidental. To determine if this interaction is biologically specific for the homologous rhizobia, the effect of cell surface polysaccharides obtained from heterologous rhizobia needs to be examined as to their ability to enhance infection. Furthermore, the structure of the active components should be determined. The molecular structures of the smallest biologically active *Rhizobium* LPS, cyclic β-2-glucan, and CPS and EPS oligosaccharide fragments need to be determined and confirmed by chemical synthesis. The ability of the smallest biologically active oligosaccharide to bind the clover root lectin trifoliin A should also be determined. Knowledge of the biologically active structure would also assist in the isolation of a perspective receptor. The effect of these cell surface polysaccharides on host gene expression, individually or in combination, needs to be investigated.

Nodule Initiation

Mutants of *Rhizobium* spp. defective in infection, nodulation, and nitrogen fixation have provided excellent tools for investigation of the developmental mechanisms of the symbiosis. The methods of molecular biology, particularly recombinant DNA techniques and transposon mutagenesis, will be exceptionally useful in delineating what signal molecules are exchanged between host and symbiont. These studies will provide insight into how rhizobia respond to an isolated signal molecule. There are two major approaches involving recombinant DNA methodologies used to identify and isolate *Rhizobium* nodulation and nitrogen fixation genes. One approach is to identify these genes by the loss of symbiotic functions in the bacterium (49, 89, 163, 186, 187, 243, 250, 281). The other approach has been to observe the acquisition of symbiotic properties in bacterial species which previously lacked them, after receipt of putative symbiotic genes (113, 114, 244, 271). Both of these experimental approaches have been particularly useful in delineating the stage of infection when nodule formation begins. This section will cover the mechanisms of nodule initiation (*noi*).

Truchet et al. (243) suggested that rhizobia produce a nodule organogenesis-inducing principle which can permeate the plant cell wall and plasma membrane. By using a Leu$^-$ auxotroph of *R. meliloti*, defective in infection thread release of bacteria, it was observed that cell proliferation and nodule formation still occur (243). The Leu$^-$ mutant-induced nodules, however, are monosomatic rather than polyploid; effective nitrogen-fixing nodules are polyploid. From this observation, Truchet et al. suggested that differentiation of the central tissue requires the presence of rhizobia in the cytoplasm (243). The term central tissue differentiation-inducing principle was coined for this signal molecule, which apparently cannot pass from cell to cell (243). Truchet et al. (244) substantiated his original observation by demonstrating that *A. tumefaciens*, carrying the *R. meliloti* Sym plasmid, induces nodule formation without the formation of infection threads. Once again the nodule tissue was free of penetrating bacteria, indicating that nodule organogenesis was triggered by a signal molecule from a distance (244). Nodule formation, therefore, is apparently independent of infection thread formation. This observation has been substantiated by the numerous investigations demonstrating the induction of cell proliferation and nodule formation without infection thread formation (89, 113, 243, 244, 250).

Another example of such a nodulation mechanism has been observed with spontaneous cell surface mutants of *R. meliloti* (89, 126). Monoclonal antibodies generated to the *R. meliloti* cell surface were used to select for mutants defective in their ability to synthesize EPS and nodulation (90, 126). These mutants do not curl root hairs or form infection threads or bacteroids, but they do penetrate the epidermis directly. Bacteria are found only in superficial intercellular spaces (89). The mutants, however, do induce cell proliferation and nodule formation (89). Bacteria are not found in the nodules. The data indicate that the signal molecule(s) which induces cell proliferation and nodule formation functions independently of infection thread formation. The data also suggest that the *R. meliloti* EPS plays a role in root hair curling and infection thread formation.

The signal molecule which induces nodule organogenesis has not been identified. Mutants which form infection threads but cannot induce nodule meristem formation would be invaluable for characterizing the signal molecule. Furthermore, the signal which induces changes in ploidy numbers and differentiation of the central tissue has not been determined. Mutants need to be isolated which infect, induce nodule formation, and release from the infection thread without inducing differentiation of the central tissue. These mutants should help to identify the signal molecule(s) involved at this step of the symbiosis. This work would require careful electron microscopic examination of the nodulation characteristics of these mutants and an examination of the mutant protein profiles and cell surface polysaccharides. Even though these investigations will require much time and effort, they could prove to be very beneficial in understanding these mechanisms.

Infection Thread Formation

The infection thread is a tubular structure of host origin in which rhizobia traverse the root hair and pass through the cortical cells. Once released from the infection thread and encapsulated in a host-derived membrane (peribacteroid membrane), the rhizobia differentiate into bacteroids. These infected plant cells continue to differentiate into an effective nitrogen-fixing nodule. Reportedly, legumes can be infected and nodulated by rhizobia without root hair curling and infection thread formation (45, 245). Our discussions, however, will be focused on the communication involved in the infection of legumes via infection thread formation.

Electron microscopy provides an excellent tool for investigating the infection of legume root hairs by rhizobia. Infection thread formation is initiated at a point where curled or branched root hairs surround the rhizobia (19, 39, 249). Invagination of the root hair cell wall is not observed (19, 39). Host cell wall material is deposited internally to the point where the rhizobia are entrapped (39). This suggests that the host cell wall is degraded at this point and a new cell wall layer is deposited with the formation of the infection thread. Degradation of the host cell wall may be accomplished by either the host or the bacteria. Ljunggren and Fahraeus (156, 157) suggested that a *Rhizobium* polysaccharide induces PGA-degrading enzyme activity in the corresponding host. The role of host polgalacturonases in the invasion of root hairs has not been conclusively demonstrated (151, 156, 157). As discussed previously, *R. trifolii* cell surface polysaccharides (CPS, LPS, β-2-glucans, and EPS oligosaccharide fragments) enhance the number of infection threads and nodules formed on white clover. These observations suggest that *Rhizobium* cell surface polysaccharides function as signal molecules which induce host responses for infection thread formation.

Calcofluor and cellufluor are fluorescent stains which bind to β-linked polysaccharides. The absence of calcofluor-white fluorescence staining of mutant strains of *Rhizobium* spp. suggests that they are defective in their cell surface (89, 250). Such mutant strains are defective in the formation of infection threads but do induce nodule organogenesis. Extracellular β-2-glucan has been implicated in infection thread and nodule formation in white clover (1, 110). It is possible that β-2-glucans may be one of the signal molecules necessary for the formation of infection threads.

Correlations among the addition of *Rhizobium* cell surface polysaccharides, infection thread formation, and polygalacturonase activity need to be established. Polygalacturonases have been shown to induce phytoalexin synthesis and other plant defense responses in numerous plants. If polygalacturonases are involved in infection thread formation, the rhizobia need a mechanism whereby they can control the host defense responses and still induce the necessary levels of polygalacturonases necessary for cell wall degradation.

Bacterial Release from the Infection Thread

Once the infection thread has traversed through the root hair into the cortical regions, it releases the bacteria into the nodule meristem. The bacteria are enveloped in the plant derived peribacteroid membrane. Mutants defective in bacterial release from the infection thread have been isolated (187, 233, 243). Mutants of *B. japonicum* which are not released from the infection thread are degraded within the infection thread (187). This suggests that bacterial release is not a passive process, but rather requires a mechanism initiated by either host or symbiont for proper release into the cortical cell tissue. Characterization of the signal molecule(s) that is exchanged will be problematic due to the difficulties associated with examining this phenomenon. A comparison of wild-type proteins and wild-type nodule proteins with those produced by mutants may be beneficial. Ploidy levels of the nodule meristem may play a role in this process. Nodule tissue is not polyploid when inoculated with mutants defective in bacteroid release from the infection thread (243).

Nodule-Specific Host Proteins

Leghemoglobin. Leghemoglobin is an oxygen-binding hemoprotein found in the host cytoplasm of legume nodules (14, 32, 91). The presence of leghemoglobin in the nodule is essential for nitrogen fixation. Nitrogenase is oxygen sensitive, and leghemoglobin binds oxygen to reduce the oxygen tension in the nodule. Leghemoglobin releases oxygen to metabolically active bacteroids at a concentration that will not damage the nitrogenase complex but will still allow bacteroid respiration. The globin apoprotein is synthesized by the legume host and is specifically induced upon infection of the plant by rhizobia (14, 32, 257). The heme portion of leghemoglobin is synthesized by the rhizobia. Heme biosynthesis in *B. japonicum* is stimulated under microaerophilic conditions (14). This observation suggests that heme biosynthesis in the nodule is due to the microaerophilic conditions that one would expect to occur in a nodule (14) and not in response to a specific signal molecule. Furthermore, mutants defective in heme synthesis (*pop*$^-$) are able to induce leghemoglobin synthesis (32). This demonstrates that the heme portion of leghemoglobin does not specifically induce the synthesis of the globin apoprotein.

The mechanism by which the infecting rhizobia induce the synthesis of leghemoglobin has not been determined. The time course for the induction of leghemoglobin mRNA has been established. RNA dot blot hybridizations demonstrate that leghemoglobin mRNAs began to appear at very low level in 3- to 5-day-old *B. japonicum*-infected soybean root tissue (91). Leghemoglobin mRNA synthesis increases dramatically after 11 days of infection (91). At this point soybean nodules are just becoming visible. Ineffective strains of *B. japonicum* (Nod$^+$ Fix$^-$) also induce leghemoglobin mRNA (91). The level of leghemoglobin, however, is reduced in comparison to the wild-type levels. A temporal relationship between leghemoglobin mRNA induction and the stage of *Rhizobium* infectivity (i.e., *roa*, *hac*, *inf*, *nod*) needs to be addressed. Further experiments to determine the time of leghemoglobin mRNA synthesis with respect to inoculation should microscopically address the stage of infection. *Rhizobium* mutants defective in nodulation could be beneficial in elucidating where in the infection process leghemoglobin synthesis is induced. It is apparent from this discussion that the signal molecule(s) that induces leghemoglobin synthesis is unknown. It is not known whether rhizobia constituitively produce the signal molecule(s) which induces leghemoglobin mRNA synthesis, or whether the signal molecule(s) is made in response to a certain stage of infection.

Nodulins. Nodulins are nodule-specific proteins encoded by the host plant. Nodulins have been isolated from pea (32), alfalfa (141), and soybean (91, 148) nodules. Two different categories of nodulins have been suggested (257). Common nodulins (c-nodulins) are expected to be common to all nodules and are involved in supporting the process of nitrogen fixation. Nodulins involved in carbon and nitrogen metabolism of the nodules have been termed s-nodulins (257). These nodulins are species specific. In soybean, a nodule-specific uricase, distinct from the root uricase, has been isolated (nodulin-35) (21). The number and size of nodulins isolated from alfalfa nodules are different from those isolated from either pea or soybean (32, 91, 141). At least one alfalfa nodulin is antigenically cross-reactive with the nodulins found in pea and soybean (141). This suggests that some of the nodulins could share common physiological properties. Mutants of rhizobia, defective in nitrogenase synthesis, induce nodulin mRNA synthesis and nodulin protein synthesis (14, 32, 91). The nodule levels, however, are reduced in ineffective tissue. The time course for the induction of soybean nodulin mRNAs is practically identical

TABLE 3. Signal molecules involved in the *Rhizobium*-legume symbiosis

Identified signal molecule	Possible or unidentified signal molecule	Physiological response	Reference(s)
SBL		Induces nodulation competence, in *B. japonicum*	101–103a
	Cowpea root factor(s)	Enhances cowpea nodulation	22–24
	Soybean/kidney bean root extract	Induces *R. fredii* promoter activity	192
Luteolin		Induces *R. meliloti* and *R. trifolii nod* promoter activity	181, 122a; S. Long and B. G. Rolfe, personal communication
Cyclic β-1,2-glucan		Increases clover root hair infection	1, 110
LPS		Increases clover root hair infection	67
CPS		Increases clover root hair infection and enhances cowpea nodulation	2, 24
EPS oligosaccharide fragment		Increases clover root hair infection	2
Acidic EPS		Root hair curling (Hac)	86, 122, 231, 273, 274
	R. trifolii culture extract protein	Clover root hair curling	86
CPS		Clover root hair curling	86, 122
	Factors released upon attachment/colonization	Cortical cell division	49, 89, 113, 114, 163, 186, 187, 243, 244, 250, 271, 281
	Factors released after infection	Subsequent nodulation inhibited	30, 40, 138, 198
	Factors released after infection	Leghemoglobin and nodulin synthesis	14, 21, 32, 91, 141, 148, 257

to that observed for leghemoglobin (32). How nodulins are induced by infecting rhizobia has not been determined.

Concluding Remarks

The establishment of the nitrogen-fixing *Rhizobium*-legume symbiosis is a complex developmental process. The molecular events necessary for establishing a symbiotic relationship have not been completely determined. A summary of both the potential and identified signal molecules involved in the *Rhizobium*-legume symbiosis is given in Table 3. The exchange of signal molecules between the host legume and rhizobia is necessary for the initiation, development, and maintenance of the symbiosis. Recognition of a compatible partner between rhizobia and host is apparently the first step in the interaction. Recognition is most likely not a single step but rather the culmination of a series of events. The failure of homologous rhizobia or host to recognize or promptly respond to a signal molecule could prevent the establishment of an effective symbiotic relationship.

Lectins have been implicated as mediators of specific recognition and attachment of homologous rhizobia to host plants. Until recently, however, it had not been demonstrated that rhizobium-lectin interaction had an effect on nodulation. The available data suggest that recognition of the galactose-specific soybean lectin by *B. japonicum* induces de novo synthesis of proteins necessary for nodulation (103a). Binding host lectins by homologous rhizobia may function as a signal to the compatible rhizobia to induce the expression of genes necessary for nodulation. Rhizobial cell surface polysaccharides (CPS, LPS, β-2-glucans, and EPS oligosaccharide fragments) enhance infection thread formation and nodulation of host plants (1, 2, 24, 67). These cell surface polysaccharides apparently function as signals to the host plant to prepare the plant for infection and nodulation. It should be determined whether enhanced infection and nodulation are specific to homologous rhizobia by pretreating host plants with cell surface polysaccharides from rhizobia. Furthermore, the effect of these cell surface polysaccharides on host gene expression needs to be examined. A comparison of host genes (induced by cell surface polysaccharides) with those genes induced upon infection (i.e., nodulins) would help to determine the physiological function of the rhizobial cell surface polysaccharides. Theoretically, genes could be induced by rhizobia which promote or hinder infection and nodulation.

As mentioned previously, recognition is most likely not a single step but the culmination of a complex series of events. Recognition of the plant cell surface may induce physiological changes in homologous rhizobia. The rhizobial cell surface can be modified by host root polysaccharide-degrading enzymes (69, 230) or by host root-produced factors (24). These modified cell surfaces may exhibit greater biological activity than the cell surface polysaccharides isolated from pure culture. Subsequent investigations, therefore, should consider the effect host roots or purified root cell surface structures have on rhizobia and the physiological properties these modifications exhibit in establishing the symbiosis.

The exchange of signal molecules between host and rhizobia is not limited to the initial stages of the interaction. The inability of the host or bacterium to respond appropriately at a particular developmental step of the symbiosis may result in the termination of the association. Interaction between the partners, therefore, is a necessity for the successful establishment of the symbiosis. The complexity of the symbiotic relationship has made it difficult to investigate the molecular events required for establishing the symbiosis. Bioassays have been a useful tool to delineate the molecular events associated with recognition and infection. Recombinant DNA methodologies may be particularly useful in elucidating the molecular events of the symbiosis

beyond the initial stages of recognition and infection. Transposon mutants defective in various stages of the symbiosis could be extremely useful. Comparisons of the mRNA and protein profiles of these mutants with the wild type will be necessary. Furthermore, the exact developmental stage in which these mutants are defective in the symbiosis will have to be determined. The focus of this research should be the identification of the signal molecules involved and the physiological effect they have in establishing and maintaining the symbiosis.

CONCLUSION

The biological systems covered in this review are the best characterized and provide different examples of signal molecules involved in plant-microbe interactions. Table 4 lists several of the signal molecules in the plant-microbe interactions reviewed and the physiological responses to these signal molecules. It is evident from Table 4 that the mode of interactions can be common to several associations, but individual interactions may be species specific. For example, fungal extracellular glycoproteins have been implicated as mediators of compatibility/incompatibility determinations in race-specific pathogen interactions. Lectins are apparently involved in many plant-microbe interactions. SBL can induce nodulation competence in its symbiotic partner, *B. japonicum*, and inhibit growth of the fungal pathogen *P. megasperma* f.sp. *sojae*. Plant lectins have also been suggested to be specifically involved in establishing the nitrogen-fixing *Rhizobium*-clover symbiosis. β-2-Glucans isolated from both *Rhizobium* and *Agrobacterium* spp. appear to play a role in the infection of their respective hosts.

The response of the host/microbe to a signal may be general or very specific. One might predict a priori that general responses, such as plant defense mechanisms, would be caused by a general class of signal molecule found in a wide number of pathogens. An example of such a signal molecule would be the cell wall oligosaccharides of fungal pathogens or the endogenous elicitor from plant cell walls. These factors are polysaccharides/oligosaccharides. On the other hand, one could predict a priori that specific responses would be mediated by factors produced only by the respective plant/microbe partner. An example of this would be the specific interaction of host lectin with the respective *Rhizobium* symbiont.

Recognition of a cell or molecular signal or both primarily occurs at the cell surface, and exchange of signal molecules occurs throughout the plant-microbe interaction. This fact is probably best exemplified by the *Rhizobium*-legume symbiosis. Molecular signals may interact with a cell surface receptor, causing the expression of a secondary signal within the cell. This secondary signal or message could then directly regulate the expression of genes involved in the interaction. The message (signal) could also be transported across the cell wall and plasma membrane directly. An example of this type of molecule is the T-DNA of *Agrobacterium* spp. Once the T-DNA is integrated into its host's genome, expression of the T-DNA genes by the host results in the establishment of crown gall disease.

It is evident from Table 4 that many of the signal molecules involved in plant-microbe interactions are polysaccharides (e.g., LPS, β-2-glucans, β-3,6-glucan, oligogalacturonides) or polysaccharide-binding proteins (lectins). Recently, it has become evident that plant cell wall polysaccharides (oligosaccharins) can have important biological regulatory functions. The data suggest that polysaccharides and lectins could function together as an important regulatory mechanism in plants. Therefore, polysaccharides and lectins not only function in plant-microbe interaction but also may play a critical role in plant metabolism. By this theory, it is conceivable that during the evolution of the plant-microbe association the microbe adapted to this natural regulatory scheme. This suggests that polysaccharide-protein interactions are not just involved in recognition or cell-cell adhesion, but can specifically induce gene expression which affects the development of the plant-microbe interaction.

Identification of the signal molecules involved in the induction of host defense responses, parasitic stages, or crown gall disease or in establishing the nitrogen-fixing *Rhizobium*-legume symbiosis is not complete. Additional

TABLE 4. Signal molecules involved in plant-microbe interactions

Identified signal molecule	Physiological response	Reference(s)
Arachidonic and eicosapentaenoic acids	Phytoalexin synthesis	23, 36, 37, 199
Fungal glycopeptide/ethylene	Increases HPRG[a] content of plant cell walls	87, 88, 240, 241
Fungal extracellular glycoproteins	Compatibility/incompatibility determination	12
SBL	Induces nodulation competence in *B. japonicum*	101–103
Galacturonic acids	Induces encystment and hyphae in *Phytopthora* sp.	99, 124
Oligogalacturonide (DP = 2–6 to DP = 20–30)	Proteinase inhibitors I and II	31, 213, 262, 269
Oligogalacturonide (DP > 10)	Phytoalexin synthesis	58, 146, 147, 248
Cyclic β-1, 2-glucan	Agrobacterium attachment and/or virulence	207
Cyclic β-1, 2-glucan	Increases clover root hair infection and nodulation	1
Hepta-β-glucoside alditol (β-3, 6-glucan)	Phytoalexin synthesis	11, 15, 223–225
LPS	Increases clover root hair infection	67
CPS	Increases clover root hair infection and cowpea nodulation	2, 24
CPS	Clover root hair curling	86, 122
Acidic EPS	Clover root hair curling	86, 122, 231, 273, 274
T-DNA/indoleacetic acid and cytokinin	Crown gall disease	4, 48, 123, 149, 197, 216, 238, 268
Opines	Conjugal transfer of Ti plasmid	118, 197
Acetosyringone, hydroxyacetosyringone	Induction of *vir* promoter	234a
Luteolin	Induction of *nod* genes	122a, 181; S. Long and B. G. Rolfe, personal communication

[a] HPRG, Hydroxyproline-rich glycoprotein.

biochemical, physiological, and genetic research is needed to further understand the mechanisms of plant-microbe interaction.

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